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Collaboration across the Pacific: China-U.S. partnership seeks to improve Earth system predictions at high resolution

There is a pressing need to develop a new advanced modeling framework for high-resolution multiscale Earth system predictions. We have already seen that climate variations impact the likelihood and intensity of extreme weather, including tropical cyclones, heat waves, winter storms, droughts, floods, and coastal sea-level rise. Such events have profound effects on human well-being, particularly as it relates to agriculture, energy use, industrial activity, marine ecosystems, and coastal sustainability, with economic impacts in the billions of dollars.

In order to address this need, Qingdao National Laboratory for Marine Science and Technology (QNLM), Texas A&M University (TAMU), and the U.S. National Center for Atmospheric Research (NCAR) are collaborating to establish the International Laboratory for High-Resolution Earth System Prediction (iHESP). This international collaboration has the potential to yield enormous social, economic, and environmental benefits, making it an extremely valuable effort for policymakers and stakeholders. The new laboratory will play a fundamental role in moving Earth System science and prediction forward by combining the expertise of these three renowned research institutions to pursue transformational efforts in the development of high resolution Earth System models.

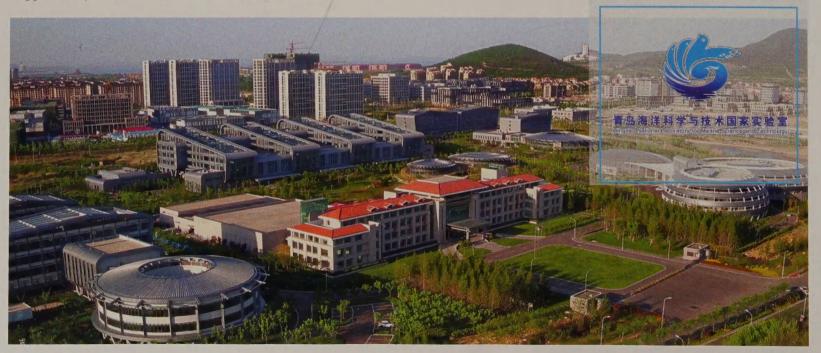
The new modeling framework and products that iHESP intends to develop will be critical to formulating solutions for risks associated with rapidly changing environmental conditions across the planet, including those associated with climate variability on subseasonal to decadal timescales. iHESP seeks to provide reliable information at both global and regional levels, thereby helping decision makers around the world to implement more effective policies.

QNLM, a hitherto unique national laboratory, was launched in 2015 with Professor, Lixin Wu, Fellow of the Chinese Academy of Sciences, as its director. A comprehensive, world-class marine science institution, QNLM pools innovative resources and teams for original research, to boost the nation's capacity for innovation and to take the lead in marine science and technology. Jointly supported by national and local government, QNLM focuses



Lixin Wu, Director of QNLM

on basic and applied research, and especially the development of cutting-edge technology. The research areas cover ocean dynamics and climate change, marine life processes and bioresource utilization, benthic processes and mineral resources, evolution and protection of marine ecological environments, extreme environments and resources in deep sea and polar oceans, and marine technologies and equipment. QNLM has launched one of the world's fastest marine research computers, and created a research vessel sharing system, including the manned submersible Jiaolong. Sticking to the principles of "openness, mobility, collaboration, sharing," and as part of its internationalization strategy, QNLM vigorously advances the construction of a global collaborative innovation network. In cooperation with Australia's Commonwealth Scientific and Industrial Research Organization (CSIRO), QNLM has established the Centre for Southern Hemisphere Oceans Research (CSHOR), to collaborate on the southern hemisphere oceans and Antarctic observation and research. To respond to the many challenges facing ocean science today, ONLM actively participates in building the international network for cooperation and exchange, holding the Global Ocean Summit to promote the sharing of knowledge, experience, and infrastructure.



TAMU, located in College Station, Texas, and established in 1876, is the flagship school of the Texas A&M system. The fourth-largest university in the U.S. and the largest in Texas, it is one of 17 U.S. universities to be designated a land-grant, seagrant, and space-grant university. TAMU is also one of the few universities in the United States to have a college of geosciences that encompasses a broad range of disciplines in Earth system science, including oceanography, atmospheric sciences, geology and geophysics, and geography, as well as the International Ocean Discovery Program (IODP)—a U.S. National Science Foundation-funded international marine research collaboration dedicated to advancing scientific understanding of the Earth through drilling, coring, and monitoring the subsea floor. Together, these academic departments and research centers have brought TAMU a wide range of talents and capabilities in Earth system science, positioning it extremely well to deal with challenges in integrated research.

NCAR, which is the U.S. National Science Foundation's oldest and largest federally funded research and development center, is one of world's premiere centers in atmospheric and Earth system science. Established in 1960, NCAR's mission is to understand the behavior of the atmosphere and related Earth and geospace systems; to support, enhance, and extend the capabilities of the university community and the broader scientific community, nationally and internationally; and to foster the transfer of knowledge and technology for the betterment of human life. NCAR is committed to the continued advancement of Earth system modeling, improved predictive capabilities, and more effective applications of these advances to societal needs. Through this new international laboratory, and in collaboration with the broader research community, NCAR will be able to accelerate its progress on these strategic objectives, thereby enhancing its ability to bring relevant and objective information to national and international decisions on mitigation, adaptation, resiliency, and sustainability.



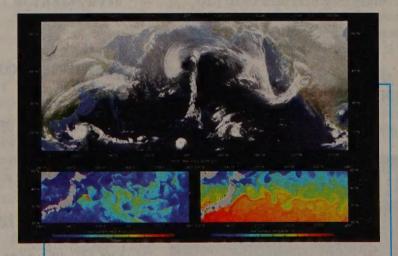
QNLM, TAMU, and NCAR representatives signing the iHESP memorandum of agreement.

iHESP is anticipated to begin operations in January 2018. Its overarching objective is to accelerate efforts in: (1) high-resolution ocean and Earth system model development; (2) high-resolution ocean and Earth system simulation and prediction; and (3) advancing scientific understanding of interactions among different Earth System components across different space and timescales.

The specific science goals of iHESP for the next five years are as follows:

- 1) Assess and quantify the role of mesoscale ocean eddies and their interactions with the atmosphere and sea-ice in climate variability, predictability, and prediction by carrying out an unprecedented ensemble of present and future climate simulations at high resolution;
- 2) Develop a new advanced modeling framework for highresolution regional and global Earth system predictions at subseasonal to decadal timescales by focusing on:
- Advancing ocean component of Earth system models with improved upper-ocean mixing processes, including surface wave and tidal effects, as well as other unresolved small-scale dynamics;
- Developing a new online coupled data assimilation capability for high-resolution regional and global Earth system models; and
- Enhancing the Community Earth System Model (CESM) coupling software framework by developing a set of online nesting tools for dynamical downscaling through nesting of a regional CESM version within the global CESM.

Today, as innovative resources are actively exchanged among the global marine science community at an unprecedented rate, open science and collaboration have become an important mode for international science and technology innovation and development. It is believed that iHESP will quickly develop into a world-class research center for Earth system modeling and prediction, greatly benefiting the global marine science community and all humanity, through gathering high-caliber talent, pooling complementary resources, and carrying out innovative research. It is also expected that iHESP will work with such international programs and initiatives as Future Earth, the International Geosphere Biosphere Program (IGBP), and the World Climate Research Program (WCRP) to provide scientific guidance and management strategies for climate prediction.



A snapshot taken from a 9-km coupled regional climate model simulation of North Pacific storm systems interacting with the Kuroshio Current and its eddies, conducted as a collaboration between TAMU and QNLM scientists. The top panel shows simulated outgoing longwave radiation (OLR) (Wm-2); lower-left and lower-right panels show the net surface heat flux (SHF)(Wm-2) and sea-surface temperature (SST)(C) over the Kuroshio Extension region. Ocean mesoscale eddies are clearly visible in the SHF, indicative of strong feedbacks between ocean mesoscale eddies and atmosphere. Wm-2, watts per square meter; C, centigrade.

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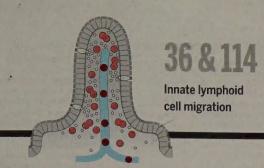
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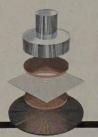
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Illustration of gut microbial species assembling into the shape of a cancer awareness ribbon. Cancer patients with an abundance of different gut bacteria have better survival outcomes

after checkpoint inhibitor immunotherapy, whereas patients with depleted gut flora respond poorly to this treatment. Thus, modulating the microbiome may provide hope for improved cancer patient responses. See pages 32, 91, 97, and 104. *Illustration: V. Altounian*/Science

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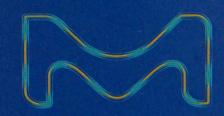
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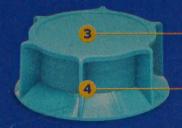
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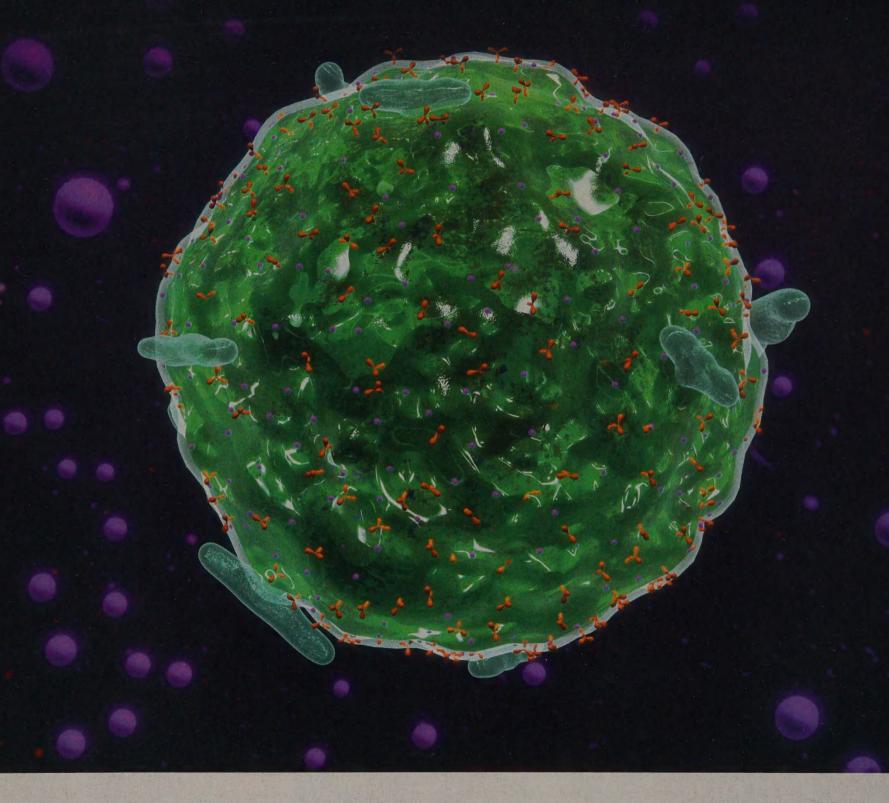








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NAAAS

Progress on reproducibility

deas supported by well-defined and clearly described methods and evidence are one of the cornerstones of science. After several publications indicated that a substantial number of scientific reports may not be readily reproducible, the scientific community and public began engaging in discussions about mechanisms to measure and enhance the reproducibility of scientific projects. In this context, several innovative

steps have been taken in recent years. The results of these efforts confirm that improving reproducibility will require persistent and adaptive responses, and as we gain experience, implementation of the best possible practices.

A framework has been developed to promote transparency and openness in scientific publications—the Transparency and Openness Promotion (TOP) guidelines (http://science. sciencemag.org/content/348/ 6242/1422.full). They cover key principles that apply to many scientific fields, although they were developed primarily by social scientists. The editors at Science have adjusted practices based on these policies and have gained experience with many of these issues. We fully support the principles behind these guidelines, including the centrality

and benefits of transparency, as captured in our editorial principle that "all data and materials necessary to understand, assess, and extend the conclusions of the manuscript must be available to any reader" of *Science* and the *Science* family of journals. Our editorial policies now contain specific statements for each TOP guideline category. In some cases, we include the possibility of granting specific exceptions but insist that these circumstances be discussed with our editors early in the manuscript evaluation process to allow for thoughtful examination.

Another approach to assess reproducibility involved an experimental program that attempted to replicate selected findings in cancer biology by groups not involved with the original studies (see https://elifesciences.org/collections/9b1e83d1/reproducibility-project-cancerbiology). Although some findings were largely reproduced, in at least one case (which was published in *Science*), the key finding was not. Yet, the initial results have

been utilized and extended in published studies from several other laboratories. This case reinforces the notion that reproducibility, certainly in cancer biology, is quite nuanced, and considerable care must be taken in evaluating both initial reports and reported attempts at extension and replication. Clear description of experimental details is essential to facilitate these efforts. The increased use of preprint servers such as bioRxiv by the biological and

biomedical communities may play a role in facilitating communication of successful and unsuccessful replication results.

Over the past year, we have retracted three papers previously published in Science. The circumstances of these retractions highlight some of the challenges connected to reproducibility policies. In one case, the authors failed to comply with an agreement to post the data underlying their study. Subsequent investigations concluded that one of the authors did not conduct the experiments as described and fabricated data. Here, the lack of compliance with the data-posting policy was associated with a much deeper issue and highlights one of the benefits of policies regarding data transparency. In a second case, some of the authors of a paper requested retraction after

they could not reproduce the previously published results. Because all authors of the original paper did not agree with this conclusion, they decided to attempt additional experiments to try to resolve the issues. These reproducibility experiments did not conclusively confirm the original results, and the editors agreed that the paper should be retracted. This case again reveals some of the subtlety associated with reproducibility. In the final case, the authors retracted a paper over extensive and incompletely described variations in image processing. This emphasizes the importance of accurately presented primary data.

As this new year moves forward, the editors of *Science* hope for continued progress toward strong policies and cultural adjustments across research ecosystems that will facilitate greater transparency, research reproducibility, and trust in the robustness and self-correcting nature of scientific results.

-Jeremy Berg

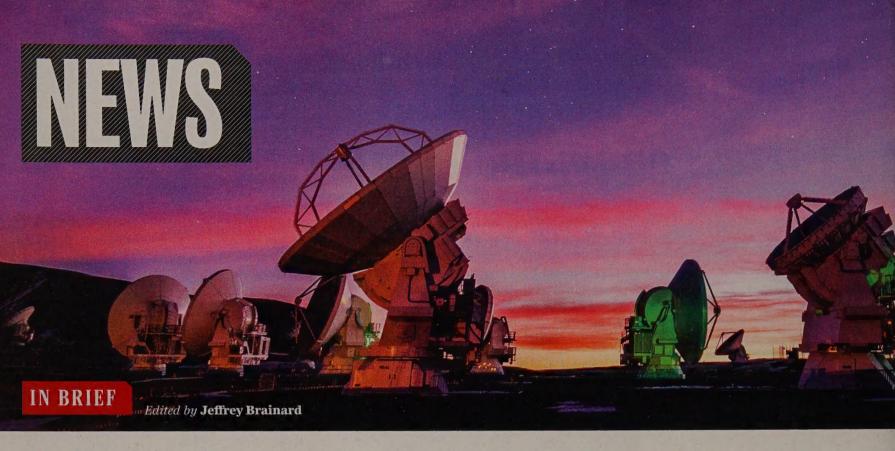


"...improving reproducibility will require...the best possible practices."



Editor-in-Chief, Science Journals. jberg@aaas.org

10.1126/science.aar8654



AREAS TO WATCH

What's coming up in 2018

umultuous political change will shape the course of science in the new year. U.S. President Donald Trump's administration is expected to continue working to dismantle science-based environmental regulations. The United Kingdom's decision to leave the European Union has posed unresolved questions about research funding and migration of scientists. And China's push to become a scientific and economic leader is sure to affect how, and where, research is done. As these broad trends play out, Science's news staff predicts specific areas of research and policy likely to be in the news this year.

A look at a monster black hole

ASTRONOMY | An international team of astronomers took a snapshot in April 2017 of the supermassive black hole at the heart of our galaxy. In the coming months, they hope to discover how well the picture came out. The Event Horizon Telescope team marshaled the forces of six radio observatories-a total of 80 dishes stretching from Hawaii to Spain to the South Pole. This multitude was needed to get sufficient resolution to image the black hole, which, though huge in mass (the equivalent of 4 million suns), is surprisingly small in volume (with a diameter less than half the distance from Mercury to the sun). After processing and correlating the data, they will obtain either a glorious silhouette of the black hole against the brilliant matter swirling around it or, as in earlier attempts using fewer telescopes, a tantalizing blur.

New clues from ancient DNA

HUMAN ORIGINS | New sources of ancient DNA should amplify the power of this molecular relic to shed light on human ancestors. Bones are the usual source of ancient human DNA, but last spring, scientists announced that they had managed to pick it up from cave sediments. That achievement may power a boom in studies tracing Neandertal, Denisovan, or modern human DNA to particular sites and artifacts, further detailing our ancestors' interactions with other archaic humans. And although most ancient DNA has come from cold climates, where it degrades slowly, results from warmer spots are emerging thanks to better sampling and analytic techniques. Ancient DNA from Africa may reveal new clues to our species's origin and diversity, and data from South America and Asia could uncover the paths humans took as

they settled the globe. Other studies will zero in on the origins of Italians, Jews, Palestinians, and people from India.

A test for quantum computing

COMPUTER SCIENCE | Physicists may soon reach a major milestone in the decadeslong quest to build a quantum computer, which instead of flipping ordinary bits would rely on subtler phenomena, such as the interference of quantum waves, to perform calculations. Researchers at Google and other laboratories are racing to build a quantum computer big enough to solve a test problem that would overwhelm ordinary computers. Such a demonstration of "quantum supremacy" would prove that the devices can do things that conventional computers cannot. But it will be years before quantum computers have enough power and reliability to achieve some of the much-hyped practical applications of such machines-including cracking current internet encryption schemes.

A big health study gets rolling

BIOMEDICINE | This spring, the U.S. National Institutes of Health will hit the launch button on one of its most ambitious projects ever: a long-term study of at least 1 million Americans that will explore the interplay among genes, lifestyle, the environment, and health. The projected 10-year, \$4 billion All of Us study, proposed by then-President Barack Obama in 2015 as part of a push for personalized medicine, has already enrolled more than 15,000 participants for pilot testing. By the end of this year, study leaders aim to sharply increase that number



and ensure that at least 50% of volunteers are from groups underrepresented in biomedical research. Persuading large numbers of people from minority groups that have been ignored or even mistreated historically in biomedical studies to share their medical records and genetic data with researchers may be a tall order.

Epidemics make a return

PUBLIC HEALTH | Epidemics are hard to predict, but recent years have seen a resurgence of old foes, and it is likely the world will witness more in 2018. Wherever conflicts or other crises lead to a breakdown of sanitation, vaccination, or the health system generally, defeated diseases return. There are fears of a yellow fever outbreak in Nigeria, where widely scattered cases have popped up, suggesting transmission is already taking place across a large area. Diphtheria is staging a comeback among the Rohingya refugees in Bangladesh and

which had not seen cases in a quartercentury. The cholera outbreak in Yemen is the biggest in modern history, with 1 million suspected cases in 2017 alone. cine are limited, but a new cholera vaccine manufacturer may help ease the shortage.

Next steps for Brexit

POLICY | The United Kingdom's decision to leave the European Union in 2019 has raised so many unanswered questions, including its effects on the research world, that we're highlighting it for the second year in a row. Negotiations with Brussels in the coming year will be critical in resolving issues such as trade, immigration, and the terms under which the United Kingdom might participate in EU research funding programs. December brought some good news for U.K. research: EU scientists already living in the United Kingdom can

sickening hundreds in war-torn Yemen, Stockpiles of cholera and yellow fever vac-

remain there post-Brexit, and the United Kingdom will remain in Horizon 2020, the EU research program, until it ends in 2020.

Europe crafts research megaplan

FUNDING | The European Commission's next large funding program for research and innovation will start taking shape this year. The 7-year program, to begin in 2021, replaces Horizon 2020, which provided €77 billion between 2014 and 2020. National governments, industry, and academia have begun lobbying for what they want to see in the plan. But after decades of steady increases, the research budget now faces fallout from Brexit: When the United Kingdom leaves the European Union in 2019, some EU programs will see their budgets shaved as the bloc's revenues decline.

Shots may treat HIV

PUBLIC HEALTH | More than 30 antiretroviral (ARV) drugs have come to market to treat HIV, and many combinations enable infected people to live near-normal life spans. But the pills work only if taken, and many infected people have difficulty adhering to their daily regimens. Later this year, several large studies are expected to report whether injectable, long-lasting ARVs given once every 4 weeks work as well as pills taken daily. Studies of monkeys suggest injected ARVs might last 3 months. and human studies also are now examining long-acting monoclonal antibodies that target HIV. These injections could be a game changer for prevention, too, as ARVs taken by uninfected people—so-called preexposure prophylaxis-derail transmissions.

A rush to the moon

SPACE FLIGHT | President Donald Trump directed NASA to return U.S. astronauts to the moon, but it may be crowded by the time they get there. Both India and China are planning to put down robotic landers on the moon this year (the Chandrayaan-2 and Chang'e 4 missions, respectively) and five companies are vying to deposit rovers there before the end of March to claim the Google Lunar XPRIZE. Launch company SpaceX may even shoot two space tourists around the moon before NASA's giant rocket, the Space Launch System, makes its first uncrewed flight, in December at the earliest.

A court ruling on gene editing

BIOTECHNOLOGY | This year, the European Union's Court of Justice is expected to decide how to regulate crops modified with



CRISPR and similar gene-editing tools. The United States moved ahead in this field in 2016 when it green-lighted one such crop: a humble white mushroom that scientists modified by removing a short DNA sequence to prevent browning. Regulators decided the mushroom was not subject to rules on genetically modified organisms (GMOs) because, unlike previous genetic engineering techniques, gene editing does not insert foreign DNA from viruses or bacteria to alter a plant's function. The court case in Europe, where public opposition to GMOs runs high, will determine whether an existing EU regulation permits a similar exception for CRISPR-edited crops.

RNA treatments move forward

CLINICAL RESEARCH | Scientists aiming to turn RNA molecules into therapies are entering this year with new optimism, after years of frustration. RNA-based drugs could treat a host of genetic diseases by intervening in the cellular process that turns DNA's messages into proteins. But it has proved tricky to sneak these delicate RNA payloads past the body's defenses and into the desired tissue while avoiding toxic



Scientists are using double-stranded RNA, shown here, to block the expression of specific genes.

effects from their delivery vehicles. In September 2017, Alnylam Pharmaceuticals announced that its small RNA molecules silenced the gene that causes the progressive disease hereditary ATTR amyloidosis in a successful phase III trial. Ionis Pharmaceuticals recently announced that an RNA approach called antisense reduced toxic brain proteins in a phase I trial to treat Huntington disease. And several ongoing clinical trials will test messenger RNA molecules that could turn cells into factories of therapeutic proteins.



One of the Large Hadron Collider's detectors could yield evidence of new kinds of particles.

FDA targets stem cell clinics

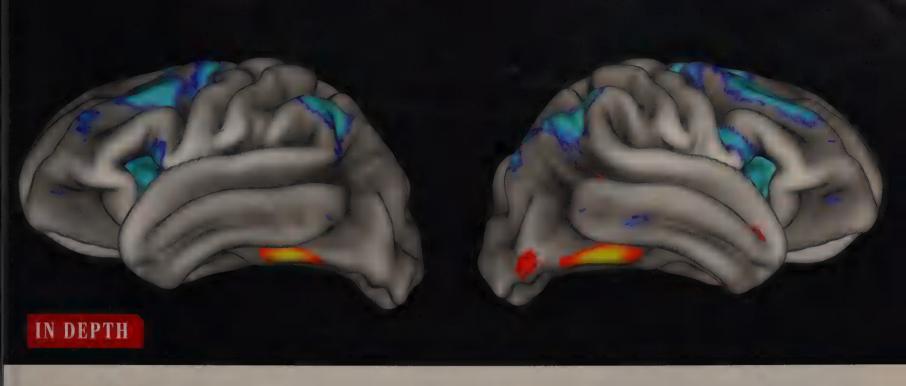
DRUG OVERSIGHT | Last year, the U.S. Food and Drug Administration (FDA) signaled that it would crack down on clinics that offer unproven stem cell treatments for conditions ranging from knee injuries to neurological disease. In 2017, the agency finalized guidelines about which products must go through its premarket approval process. FDA also issued a warning letter to one stem cell company and seized unauthorized vaccines from another. Advocates for greater oversight are now watching whether other clinics flying under the regulatory radar—which potentially number in the hundreds—will face any consequences.

Scientists run for office

POLITICS | Energized by their opposition to the science and environmental policies of President Donald Trump and the Republican-controlled Congress, many U.S. researchers have decided to take their activism to the next level by running for office. Mostly novice politicians, and almost all Democrats, they are hoping their scientific credentials will be an asset with voters. A science-friendly political action committee has already raised \$2 million for national, state, and local races-a record amount for such a cause-and helped train the candidates in the basics of running a campaign. Many will likely be derailed in crowded primaries, and none is a favorite to win in November. But their commitment to electoral politics adds a new element to the 2018 election season.

In pursuit of new particles

PARTICLE PHYSICS | Keep an eye out for clues to new particles from the world's largest atom smasher, the Large Hadron Collider (LHC) in Switzerland. In 2012, physicists there first glimpsed the Higgs boson, the last missing piece in the standard model of particles and forces. But so far, scientists haven't spotted what they want even more: new particles beyond those in the standard model. Instead of trying to see new particles directly, researchers working with the LHC beauty experiment study the decays of familiar particles called B mesons, which contain a massive bottom quark and lighter antiquark. Rates of certain decays don't quite match those predicted by the standard model. The anomalies could point indirectly to new particles flitting in and out of existence within the B mesons-a hint that scientists are eager to pursue.



NEUROSCIENCE

Watching the teen brain grow

Over a decade, researchers aim to follow 10,000 children through the challenges of adolescence

By Meredith Wadman

hya* (pronounced SHY-a), who is not quite 10 years old, recently spent an unusual day at the University of Maryland School of Medicine in Baltimore. Part of the time she was in a "cool" brain scanner while playing video games designed to test her memory and other brain-related skills. At other points, she answered lots of questions about her life and health on an iPad.

A slender Baltimore third grader who likes drawing, hip hop, and playing with her pet Chihuahua, Chya is one of more than 6800 children now enrolled in an unprecedented examination of teenage brain development. The Adolescent Brain Cognitive Development Study-or ABCD Study-will complete its 2-year enrollment period in September, and this month will release a trove of data from 4500 early participants into a freely accessible, anonymized database. Ultimately, the study aims to follow 10,000 children for a decade as they grow from 9- and 10-yearolds into young adults.

Supported by the first chunk of \$300 million pledged by several institutes at the National Institutes of Health (NIH) in Bethesda, Maryland, teams at 21 sites around the United States are regularly using MRI machines to record the structure and activity

of these young brains. They're also collecting reams of psychological, cognitive, and environmental data about each child, along with biological specimens such as their DNA. In addition to providing the first standardized benchmarks of healthy adolescent brain development, this information should allow scientists to probe how substance use, sports injuries, screen time, sleep habits, and other influences may affect-or be affected bya maturing brain.

"A lot of studies in this area are plagued by the fact that we tend to capture teenagers after they have already started to misbehave in various ways. So, the fact that we are following kids from ... before they engage in a lot of risk-taking behavior-it's going to be an incredibly rich data set," says clinical neuroscientist Monica Luciana of the University of Minnesota in Minneapolis, an ABCD Study site where she is a principal investigator (PI).

Clarifying the impacts of alcohol and drug use is a key goal for the study's leading funders: the National Institute on Drug Abuse (NIDA) and the National Institute on Alcohol Abuse and Alcoholism (NIAAA), both in Bethesda. "There is an urgency to try to address these questions," says NIDA Director Nora Volkow, the prime mover behind the study. The recent legalization of recreational marijuana in several states makes the study especially timely, she argues.

Other studies have used MRI to follow teen brain development. Europe's IMAGEN enrolled 2000 14-year-olds and scanned them at intervals over the past decade.

Combining results from 628 children's brains, this MRI scan shows regions activated as faces are viewed (yellow and orange) and other areas (blue and cyan) activated during a demanding working memory task.

And NIAAA has been funding a study of alcohol's impacts, imaging the brains of more than 800 youths once a year for 4 years. But ABCD "is going to be by far the largest," Volkow says. "It's [also] longerlasting, starts younger, and is much more comprehensive" in terms of testing.

Every 2 years, researchers will image the brain structures of Chya and other subjects, and record their neural activity as they perform tasks involving memory, rewards, and face recognition. Every year, the participants will have their height, weight, and waist measured, and answer questions designed to assess psychological symptoms. They will also be asked annually about environmental influences—things like family conflict and neighborhood crime.

The study aims to describe a normal trajectory of adolescent brain developmentsimilar to the height and weight charts in pediatricians' offices—and use that to begin to answer the many chicken-and-egg questions that correlational studies can't. For instance, people who smoke marijuana heavily beginning in adolescence or young adulthood show markedly less connectivity between the neuronal axons of the hippocampus, a brain region important to long-term memory formation and learning, than do nonusers. But were the users' brains wired differently to begin with, leading them to smoke cannabis heavily? Or did the smoking cause the changes?

"At age 9 and 10 you can get a nice clean baseline assessment on these kids," says Hugh Garavan, a neurobiologist who is the PI at the ABCD Study site at the University of Vermont (UVM) in Burlington. "Then, when someone develops psychosis at 16,

*The ABCD Study keeps participants anonymous; the families of the kids in this article allowed the use of their first names.

we can go back and look at their brains and psychological assessments at 9, 10, 11, and 12. Were there markers of risk there?"

Such markers, it's hoped, will ultimately allow much earlier diagnosis, treatment, or prevention of substance abuse, mental illness, and other conditions. A key strength of the study is that it will enroll enough children-including 800 pairs of twins-to answer questions that require large numbers of subjects, such as whether there are age windows when the brain is particularly vulnerable to use of a given drug.

The study intends to reflect the United States's socioeconomic, geographical, racial, and ethnic diversity. Planners want the study pool to be 6% Asian-American, 16% black, and 24% Hispanic. As of early January, with 8 months left in recruitment, they are falling short on Asian-American and black enrollment, at 2% and 12%, respectively, but were close to meeting the Hispanic target, at 22%. And the "other" category, which includes mixed-race kids and Native Americans, accounts for fully 11% of enrollees, more than double the organizers' 5% target.

The ABCD Study crystallized at an inauspicious time. In May 2014, when its planners held their first meeting, the National Children's Study, an NIH effort to follow 100,000 children from the womb to age 21, was collapsing as a result of daunting logistics, overambitious objectives, and a projected cost of more than \$3 billion (Science, 19 December 2014, p. 1441). "We were very, very aware, because of the Children's Study, that we didn't want to load up the Christmas tree with too many ornaments," recalls George Koob, NIAAA director since 2014.

By September 2016, under a tightly designed protocol dictated by the attention spans and wiggles of 9- and 10-year-old children, the ABCD Study was enrolling. The organizers are assuming 15% of the planned 11,500 enrollees will drop out over the decade, leaving the desired 10,000 participants. Some fear that may be optimistic, but Garavan is bullish. "Because it's a longitudinal study, we have to make sure the kids enjoy the experience," he says.

Garavan has succeeded with at least one recruit. "You had to drool into two vials, not spit. And it was fun," recalls Afton, who had his brain scanned at UVM last September, a day before his 10th birthday. "Eventually they are going to learn how to track if someone has depression or something else, or not, by just looking at their brain in an MRI machine. I thought that was cool. And I wanted to help."

Chya, for her part, left the Baltimore study site last month with a picture of her brain and an ABCD T-shirt. She plans to return at the same time next year.

ARCHAEOLOGY

Americas peopled in a single wave, ancient genome reveals

DNA from Alaskan infant suggests a long arctic sojourn

"Because they have

genome, you can really

tell a lot about when

migration happened."

Connie Mulligan, University of Florida

the whole nuclear

and where this

By Michael Price

rare smidgen of ancient DNA has sharpened the picture of one of humanity's greatest migrations. Some 15,000 to 25,000 years ago, people wandered from Asia to North America across a now-submerged land called Beringia, which once connected Siberia and Alaska. But exactly when these ancient settlers crossed and how many migrations occurred are hotly debated. Now, the oldest full genome to be sequenced from the Americas suggests that some settlers stayed in Beringia while another group headed south and formed the population from which all living Native Americans descend.

"This is an important study that sig-

nificantly narrows the subset of possibilities [for how the Americas were peopled]," David Reich, a geneticist at Harvard Medical School in Boston. "It's very exciting."

The genome comes from an 11,500-year-old infant found in 2013 at the site of Upward Sun River in central Alaska's Tanana River Basin, a

part of Beringia that's still above sea level. The infant, one of two from the site, belonged to a population that likely numbered in the low thousands, who hunted Beringia's abundant herds and gathered plants (Science, 28 February 2014, p. 961).

A team led by geneticist Willerslev of the University of Copenhagen and the University of Cambridge in the United Kingdom isolated DNA from bone powder taken from the infant's skull. The researchers sequenced the DNA repeatedly to get a virtually complete copy of the genome. They compared it to that of modern Native Americans, as well as to other ancient and living people across Eurasia and the Americas. By looking at genetic similarities and estimating how long it would take for key mutations to pop up, the researchers assembled a family tree with rough dates.

The infant's group was most closely related to modern Native Americans-but it wasn't a direct ancestor. Instead, it and modern Native Americans shared common ancestors who must have entered Beringia some 25,000 years ago, the researchers report this week in Nature. Perhaps 21,000 years ago, those ancient settlers branched into at least two groups: one that included the infant and another that gave rise to Native Americans.

That supports the idea that Asian migrants lingered in Beringia and became genetically isolated-the so-called Beringian standstill model-says anthropologist Connie Mulligan of the University of Florida in Gainesville. "Because they have the whole nuclear genome, you can really tell a lot about when and where this migration happened," she says. But Reich cautions that date estimates from a single genome are necessarily rough.

> The researchers also found that the ancient Beringian infant equally related to both the northern and southern genetic subgroups of Native Americans, implying that both descend from a single migration. The team suggests that a group headed south into North America about 20,000 years ago and

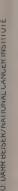
distinct subpopulations, perhaps between 14,500 and 17,000 years ago, dates that fit with previous studies.

Why did one group linger and thrive in Beringia while another took off to explore the Americas? A search for fresh resources could have spurred the migrants, Willerslev says, but so could sheer curiosity. "There were people who were happy with what they had, and there were others who looked out at the great ice caps and wanted to see what was on the other side," he says.

That's a compelling speculation, Mulligan says. "Once they got into North America, they really high-tailed it through the continent and down into South America within just a few thousand years," she says. A cultural or genetic penchant for exploration "could help explain why they were in such a hurry."

only afterward split into

Michael Price is a freelance writer in San Diego, California.





BIOMEDICINE

Cancer institute head touts big data and basic research

NCI's new director compares progress against cancer today to early strides against infectious disease

By Jocelyn Kaiser

ancer researchers had worried that President Donald Trump might choose an unconventional candidate to replace Harold Varmus as director of the National Cancer Institute (NCI) in Bethesda, Maryland. But his choice, announced in June 2017, allayed their fears. Norman "Ned" Sharpless, who was then director of the Lineberger Comprehensive Cancer Center at the University of North Carolina (UNC) in Chapel Hill, checked all the right boxes for the community: He is a physician-scientist who treats patients, he has started two biotech companies, and he runs a basic research lab focused on p16, a protein important to cell division and aging.

Sharpless, 51, who started work at NCI in October 2017, takes over from Doug Lowy, who had been acting director since Varmus's departure in March 2015. In an interview last month, Sharpless declined to lay out specific plans for the \$5.7 billion institute. But he stressed three areas: big data, basic research, and translating discoveries into devices and treatments. He's also been thinking of ways to bolster the careers of young investigators, a perennial issue for NCI's parent agency, the National Institutes of Health. This interview has been edited for brevity and clarity.

O: Why did you want to be NCI director?

A: My old boss Ron DePinho [Sharpless's postdoctoral adviser, now at MD Anderson Cancer Center in Houston, Texas], who

nominated me for the job, actually called to convince me why I should consider doing it. And I sort of stopped him in midsentence and said, "Wait, Ron, you had me at 'NCI director.'"

I really believe it's a special time in cancer research. We have this basic biological understanding and the notion that cancer is thousands of different diseases, and that has really allowed us to speed progress. We're going to remember the cancer researchers of the day the way we talk about [Louis] Pasteur and [Robert] Koch and antibiotics.

Q: Critics of IBM's use of the Watson supercomputer to guide cancer care say it merely compiles existing knowledge without producing new insights. What do you think about big data?

A: I am 100% convinced that the area of artificial intelligence and machine learning will have a huge impact on cancer research and cancer care. [At UNC], we used Watson to organize the literature related to novel therapeutics for [our] molecular tumor board that was trying to decide what drug to give a patient based on [their tumor] mutation. When you start doing a thousand patients a year, the allocation of therapy decisions gets very hard.

O: How can machine learning help make sense of cancer patient data?

A: The clinical data are the hard part. Genomic data are black-and-white compared to the clinical data in electronic

health records. Having med students reading charts and abstracting them is just too slow, and so these tools that use natural language processing to extract the records are very useful. That's going to get us a million patients that are clinically annotated and aggregated with genomic data sets.

O: Where should NCI be going with immunotherapy?

A: Around 6 years ago, one of the senior leaders of the immunotherapy movement was complaining to me about how NCI's portfolio for immunotherapy research was small and didn't have enough basic science or clinical trials. I don't think anyone could make that statement about the NCI portfolio today. ... [But] we just don't understand cell biology well enough to make full therapeutic use of immunotherapies.

Q: Are you worried about the drastic cuts to the 2018 NIH budget that the Trump administration has proposed?

A: I am obligated to support the president's budget. I will say that the congressional commitment to [NIH] has been good. The budget has gone up significantly in the last 2 years, and the [preliminary congressional] budget for 2018 looks promising as well. I think the budgetary challenges are real. Because if you consider the burden of cancer or what it costs society, even \$5.7 billion is not really enough.

Q: What are your thoughts on NIH's efforts to help young researchers?

A: One idea we've been kicking around is to give some subset of new, young investigators an extended period of R01like funding. [R01s are NIH's standard research grants.] So instead of stopping the grant at 5 years, they would submit a slightly more significant package than a standard noncompeting renewal. And if that's approved they would get 2 years extra funding.

We realize there are intense pressures on young faculty to get data together before they can get their second grant and get tenure. I'm a little worried that young investigators are becoming demoralized. We are committed to their success.

Q: Are you planning to have a lab at NIH?

A: Most of the people who worked for me at [UNC] have now gotten their own jobs. But I have some knock-in mice that I produced in my lab that I'm trying to import to the NCI so that I can start a lab effort here. It's very important to me. It's just nice to think about science. It's nice to understand the problems of a working scientist.



By Eric Hand

rom the pasture to the swamp, methane emissions on Earth are the effluvia of life. So what are whiffs of the gas doing on barren Mars? Trace detections of the stuff, alongside glimpses of larger spikes, have fueled debates about biological and nonbiological sources of the gas. Last month, at a meeting of the American Geophysical Union (AGU) in New Orleans, Louisiana, NASA scientists announced a new twist in the tale: a seasonal cycle in the abundance of martian methane, which regularly rises to a peak in late northern summer.

"The thing that's so shocking here is this large variation," said Chris Webster, who leads the methane-sensing instrument on NASA's Curiosity rover. "We're left trying to imagine how we can create this seasonal variation," says Webster, who is at the Jet Propulsion Laboratory in Pasadena, California.

It is a variation on a very faint theme. Since landing in 2012, Curiosity has on 30 occasions opened a few valves to the martian night and taken a sniff of the thin, frigid air. In a small, mirrored chamber, it shines a laser through the air sample and measures the absorption at specific wavelengths that indicate methane. At the meeting, Webster reported vanishingly small background levels of the gas: 0.4 parts per billion (ppb), compared with Earth's 1800 ppb.

Where that whiff comes from is the heart of the mystery. Microbes (including those that live in the guts of cows and sheep) are responsible for most of Earth's methane, and Mars's could conceivably come from microbes as well—either contemporary microbes or ancient ones, if the methane they produced was trapped underground. But methane can also be made in ways that have nothing to do with biology. Hydrothermal reactions with olivine-rich rocks underground can generate it, as can reactions driven by ultraviolet (UV) light striking the carboncontaining meteoroids and dust that constantly rain down on the planet from space.

Now, add to the methane puzzle the seasonal variation Curiosity has detected, with levels cycling between about 0.3 ppb and 0.7 ppb over more than two martian years.

"We're left trying to imagine how we can create this seasonal variation."

Chris Webster, Jet Propulsion Laboratory

Some seasonality is expected in an atmosphere that is mostly carbon dioxide (CO_a), says François Forget, who models the climate of Mars at the Laboratory of Dynamical Meteorology in Paris. In the southern winter, some of that CO_o freezes out onto the large southern polar cap, making the overall atmosphere thinner. That boosts the concentration of any residual methane, which doesn't freeze, and by the end of northern summer this methane-enriched air makes its way north to Curiosity's location, Forget says. Seasonal variations in dust storms and levels of UV light could also affect the abundance of methane, if interplanetary dust is its primary source.

But, Webster said at the meeting, the seasonal signal is some three times larger than those mechanisms could explain. Maybe the methane—whatever its source—is absorbed and released from pores in surface rocks at rates that depend on temperature, he said. Another explanation, "one that no one talks about but is in the back of everyone's mind," is biological activity, says Mike Mumma, a planetary scientist at Goddard Space Flight Center in Greenbelt, Maryland. "You'd expect life to be seasonal."

The seasonal wiggles are a mystery within a larger mystery: claims of occasional methane spikes an order of magnitude or two higher than the background. Mumma and his colleagues reported one of the largest in 2009, when they detected spectral signs of a 45-ppb methane plume through a telescope in Hawaii. Curiosity, too, has detected a handful of spikes, to about 7 ppb. For these events, Webster favors the idea of a sudden release from a deep underground source.

Other scientists are looking skyward. Marc Fries, the cosmic dust curator at Johnson Space Center in Houston, Texas, says the source of methane spikes could be the hail of tiny meteors that falls when a planet crosses a comet's orbit and sweeps up carbon-rich dust and debris shed by the comet. Fries says that as the dust particles vaporize at altitudes of tens of kilometers, the same chemical reaction that produces methane from interplanetary dust at the surface would take place more quickly, driven by the stronger UV light at high altitudes. All the claimed methane spikes over the past 2 decades occurred within about 2 weeks of a known martian meteor shower, Fries and

During its 5-year sojourn on Mars, the Curiosity rover has repeatedly sniffed the air for methane.

his colleagues found. "It could be a cause, and it could be a coincidence," he says.

Skeptics say the atmospheric reactions may not occur quickly enough and that meteor showers don't deposit much more material than the background flux of interplanetary dust. In 2014, when Mars nearly collided with comet Siding Spring, NASA's Mars Atmosphere and Volatile Evolution Mission (MAVEN) spacecraft was watching, monitoring magnesium ions as a proxy for dust dumped in the upper atmosphere. The MAVEN team reckons the encounter put 16 tons of material into the martian atmosphere—not much more than the 3 tons of interplanetary dust estimated to fall daily, and much less than the tens of thousands of tons that Fries says are needed to make a large methane plume. "I don't see how it's possible to produce the methane abundance he needs," says Matteo Crismani, a MAVEN science team member and postdoctoral researcher at the Laboratory for Atmospheric and Space Physics at the University of Colorado in Boulder. But Fries contends that meteor showers are highly variable, and just because the Siding Spring encounter was close does not mean it was rich in dust and debris.

It happens that Fries will have a chance to test the hypothesis. On 24 January, Mars will have a close brush-less than a tenth of the Earth-moon distance—with the orbit of comet C/2007 H2 Skiff. Mumma is skeptical about Fries's idea, but he will nevertheless be watching for methane with his telescope in Hawaii in the days after the encounter. The MAVEN and Curiosity teams also plan to watch. "This is a great opportunity to test this hypothesis," Crismani says.

One spacecraft won't quite be ready to participate-even though it is best positioned overall to resolve the methane debate. In April, the European Space Agency's ExoMars Trace Gas Orbiter (TGO) will settle into its final orbit and begin science observations, mapping concentrations of methane across the planet. Atmospheric dust will probably prevent the orbiter from reaching its originally advertised sensitivity of several tens of parts per trillion, says Geronimo Villanueva, a science team member at Goddard. But he expects the TGO to approach Curiosity's sensitivity—and its ability to hunt for methane sources in space and time will be unrivaled. The "TGO will allow us to search for this molecule with new eyes." he says.

With additional reporting by Paul Voosen from the AGU meeting.

EUROPE

Germany steps up to the plate in global health

A country long held back by its past emerges as a new leader, but its own research output is still lagging

By Kai Kupferschmidt, in Berlin

hen health ministers and other top health officials from 19 of the world's largest economies were ushered into a meeting room here in May 2017, they walked right into an unfolding international crisis. A video screen showed news reports of a mystery disease, apparently a cousin of severe acute respiratory syndrome, causing respiratory illness and death. What began as a local outbreak soon engulfed multiple countries, and the officials faced difficult choices about how to respond.

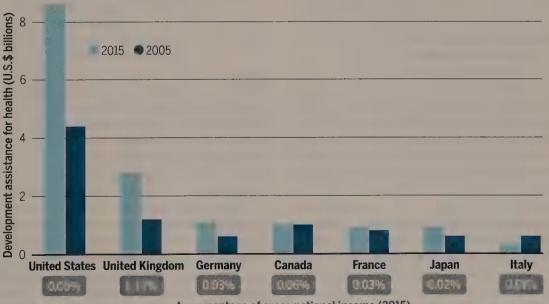
The scenario was fictitious; it began in a nation named Anycountry and was designed primarily to raise awareness about the threat of emerging diseases. But the event marked a real-world shift. It was the first-ever gathering of the G-20's health ministers, and it was organized by Germany-one of many signs that the nation's role in global health is growing rapidly.

"For a long time there was a sense that Germany wasn't contributing as much as it could," says Jeremy Farrar, director of the Wellcome Trust in London, a major private funder of medical research. But recently, the German government has embarked on several international health initiatives and has doubled its financial contributions to global health aid. As a result, the country has become a destination for major players in the field, including the Bill & Melinda Gates Foundation, headquartered in Seattle, Washington, which plans to set up an office here next year. Berlin will also host a new, international center focused on the rising threat of antimicrobial resistance (AMR), and its World Health Summit, an annual meeting here, is drawing an increasingly influential crowd.

For decades, Germany's past made it wary of taking the lead on the global stage, especially in public health, or Volksgesundheit, a label used by Nazi doctors to justify sterilizing and killing people with mental illness and epilepsy. German research in public health has long languished as well. Today, "Germany is getting rid of its post-World War II inhibitions to finally play a role that is commensurate with

Healthy growth

In addition to spearheading initiatives such as a new center to combat antimicrobial resistance, Germany has doubled its global health aid budget. It still lags far behind the United States and the United Kingdom, however.



its economic weight and its intellectual leadership," says Peter Piot, director of the London School of Hygiene & Tropical Medicine (LSHTM).

German Chancellor Angela Merkel has been a driving force. She put global health at the top of the agenda as host of the G-7 summit in 2015 and at last year's gathering of the G-20. In 2015, Merkel also became the first German chancellor to speak at the World Health Assembly, the World Health Organization's (WHO's) annual member meeting. "I have no doubt that she is committed on a personal level to this," says Farrar, who has met Merkel.

Germany's spending on development assistance for health is still below WHO's goal of 0.1% of gross national income, and far behind that of the United States and the United Kingdom. But at \$1.1 billion, it now surpasses spending by Japan, France,



German Chancellor Angela Merkel, who takes a personal interest in global health, welcomed Bill Gates in 2014.

and Canada (see graphic, p. 17). The country has championed raising WHO membership dues and is the largest contributor to WHO's new Contingency Fund for Emergencies, set up in response to the West African Ebola outbreak.

Antimicrobial resistance is a focus because it is by nature an international problem, Hermann Gröhe, Germany's minister of health since 2013, tells Science. "We can make all the effort we want in Germany, but it's all for nothing if there aren't similar efforts happening around the world." The new AMR hub will be supported by the G-20, but Germany's leadership in securing their commitment was "indispensable," says Keith Klugman, who leads AMR strategy at the Gates Foundation: "I really can't overstate the importance of this hub."

Germany has also been instrumental in setting up the Coalition for Epidemic Preparedness Innovations, a public-private coalition that aims to develop vaccines for possible future pandemics. "Germany is a latecomer, but it has been catching up fast," says Ilona Kickbusch, director of The Graduate Institute's Global Health Centre in Geneva, Switzerland. (Farrar, Kickbusch, and four other experts recently agreed to serve on an international advisory board to help the German government draw up a new international health policy strategy.)

As worries grow that the United Kingdom and the United States are becoming more inward-looking, public health leaders are happy Germany is stepping up. Whether London will remain a magnet for international talent after Brexit remains to be seen, Piot says. Gates Foundation CEO Sue Desmond-Hellmann says she is "deeply concerned" about dramatic cuts in the budget of some global health programs

> proposed by U.S. President Donald Trump. "We're extremely positive about having wealthy nations like Germany or China emerge as government spenders in this area," she adds.

> Yet Gröhe is quick to point out that Germany can't make up for a retreat by other nations. "I would warn against any expectation that Germany could somehow replace the U.S. engagement," he says. "But we can complement it." And although Merkel's Christian Democrats won the biggest share of the vote in October 2017's federal elections, attempts to form a coalition with the Liberal Democrats

and the Greens fell apart in late November 2017; it's unclear what the next government will look like and whether it will continue the country's newfound leadership.

Meanwhile, Germany's own research output in global health is still lagging. Piot says that in tallies of joint authorship, LSHTM researchers "publish more with the Netherlands, Belgium, Switzerland, or France than with Germany." A 2015 assessment by the German National Academy of Sciences also noted that countries with far smaller populations such as Canada, Sweden, and the Netherlands produced more papers on public health than Germany. "Although there are excellent individuals and institutions working in public and global health in Germany," it said, "they need increased political support, improved structures, and significant research investment." Germany's next big effort in global health may well be at home.

ASTRONOMY

Earth-based planet finders power up

Increases in precision put Earth twins in reach

By Daniel Clery

hen it comes to finding new worlds, NASA's Kepler spacecraft hogs the headlines, having racked up thousands of exoplanet discoveries since its launch in 2009. But before Kepler, the workhorses of exoplanet identification were ground-based instruments that measure tiny stellar wobbles caused by the gravity of an orbiting planet. They are now undergoing a quiet renaissance. The new generation of these devices may be precise enough to find a true Earth twin: a planet with the same mass as ours, orbiting a sunlike star once a year. That's something Kepler-sensitive to planet size, but not mass-can't do.

On 9 December 2017, the Extreme Precision Spectrometer (EXPRES) took its first view of the sky on the Discovery Channel Telescope in Arizona. And in October 2017, the Echelle Spectrograph for Rocky Exoplanet and Stable Spectroscopic Observations (ESPRESSO) began operating at the European Southern Observatory's (ESO's) Very Large Telescope in Chile. Nearly two dozen other instruments are either under construction or have recently begun service. "It's now clear that exoplanets are major part of astronomy." says astronomer Jason Wright of Pennsylvania State University in State College. "So every major observatory needs a highresolution spectrograph."

Such spectrographs spread starlight out into its array of spectral colors, which contains dark lines at wavelengths where gases in the star's atmosphere absorb light. Astronomers then look for tiny oscillating Doppler shifts in these lines over time. caused by planetary tugging.

The technique works best for a massive planet orbiting close to its star, because its gravitational tug will be stronger. As a result, early discoveries-such as the first exoplanet to be found, in 1995-were usually "hot Jupiters," giant planets in tight



orbits. Then came the High Accuracy Radial velocity Planet Searcher (HARPS) at ESO's La Silla Observatory in Chile in 2003, a second-generation spectrograph that enabled astronomers to find smaller planets in wider orbits. (It has spotted about 130 to date.)

In recent years, however, a new "transit" technique, led by Kepler, began to dominate. Kepler stared at 145,000 stars in one part of the sky and looked for dips in stellar brightness when a planet passed in front. Although Kepler has been prolific, identifying thousands of exoplanets and gauging their sizes, the planets and their stars are mostly too far away for groundbased spectrographs to determine their masses. Over the coming year, the launch of Kepler's successor, the Transiting Exoplanet Survey Satellite (TESS), along with Europe's Characterising Exoplanets Satellite (CHEOPS), will change all that. They will scour the sky for transits of nearby bright stars-perfect for ground-based follow-up. "To understand them we have to know their mass, that's absolutely fundamental," says René Doyon of the University of Montreal in Canada.

The new spectrographs should be able to weigh many of the planets found by TESS and CHEOPS. Astronomers boosted the instruments' precision in part by isolating them from mechanical and thermal noise—mounting them inside vacuum vessels that are separated from the telescope and piping in light through optical fibers.

They also improved the reference spectrum against which the slow shifts in the spectral lines are gauged.

Instruments such as HARPS rely on reference spectra produced by a thorium-argon lamps. But these produce a jumble of spectral lines of varying brightness. The new wave of spectrographs, including ESPRESSO and EXPRES, instead use laser frequency combs, which duplicate a single spectral line from a laser to form an ultra-

"To understand [exoplanets] we have to know their mass, that's absolutely fundamental."

René Doyon, University of Montreal

precise reference grid, with lines of equal brightness spaced at regular intervals.

With these improvements, says lead scientist Francesco Pepe of the University of Geneva in Switzerland, ESPRESSO aims to measure stellar movements as slow as 10 centimeters per second (roughly the speed of a giant tortoise). That would be a factor of 10 better than HARPS, and is exactly the motion expected to be caused by an Earth twin in an Earth-size orbit around a sunlike star.

At this sort of precision, pulsing gases in the star's atmosphere can swamp the

signal of any wobble. With some of the new spectrographs, researchers hope to disentangle the wobble from the noise by comparing spectral shifts across a range of wavelengths. But Pepe expects the noise to limit ESPRESSO to planets that are about three or four times as heavy as Earth. Only if the star is much less massive and more susceptible to a planet's tug might ESPRESSO see Earth-size planets.

Other spectrographs aim for increased sensitivity to small planets by focusing on some of the smallest stars: red dwarfs. Red dwarfs emit most of their light in the infrared. Yet Earth's atmosphere allows only a narrow range of near-infrared wavelengths to reach the ground, posing a challenge for instrument builders. "There is a big push to develop instruments," says Doyon, who leads a team building the Near-InfraRed Planet Searcher to work alongside HARPS on ESO's 3.6-meter telescope in Chile.

The new instruments could even enhance transit studies. Besides searching for the spectral wobbles, they might see additional spectral lines caused by starlight passing through the atmosphere of a transiting planet. Transit spectroscopy with existing instruments has already revealed gases such as water and carbon dioxide in exoplanet atmospheres, and astronomers are now using it to look for signs of life (*Science*, 3 November 2017, p. 578). If the new spectrographs can characterize planets as well as find them, the renaissance could become a revolution.



CHEMICAL MARTYRS

Three decades after Iraq unleashed chemical weapons on Iran, scientists there are unraveling the long-term effects

By Richard Stone, in Tehran

n a chilly morning in September 1987, in the waning days of the Iran-Iraq War, an 18-year-old Iranian soldier named Seyed Naser Emadi drove from a battlefield in northwestern Iran to a hospital in Nagadeh, a city a couple hours away. Crammed in the back of his Land Rover were four soldiers, moaning, vomiting, and coughing. "I'll never forget the sound of their breathing," Emadi says. "It was rattling, raspy." The terrified young men did not know what was happening, and Emadi had no idea how to help them.

They complained about the cold, so Emadi rolled up his window. The air in the vehicle became stifling, and a strong chemical odor wafted from the men's uniforms. Emadi grew nauseous and dizzy, but he made it to the hospital. There, he tried to rouse the nearest victim, whose face and hands had large blisters. The young man would not wake up. As Emadi and a hospital aide carried the victim out of the vehicle, a blister on the man's arm burst, and fluid splattered on the back of Emadi's hand. A couple hours later, he felt a burning sensation and his own hand began to blister.

The experience set Emadi on the path to becoming a medical researcher. (He is now a dermatologist at Tehran University of Medical Sciences and a volunteer with Doctors Without Borders.) It also highlighted one of the cruelest practices in the 1980-88 conflict, which also featured child soldiers and trench warfare. For the first time ever on a battlefield, nerve agents including sarin and tabun were unleashed by Iraqi forces. "These are the most toxic chemical warfare agents ever developed," says Jonathan Newmark, a neurologist in Burke, Virginia, who has helped develop countermeasures against such weapons at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) in Edgewood, Maryland, and other agencies. And on scores of

occasions, Iraq shelled soldiers and villagers with sulfur mustard—the chemical that afflicted Emadi and his passengers.

After the war, Iraq—pressured to own up to the attacks—acknowledged that it had "consumed" 1800 tons of mustard, 600 tons of sarin, and 140 tons of tabun. All told,





Seyed Naser Emadi as a teenage soldier in the Iran-Iraq War, where he witnessed chemical attacks, and at a meeting of survivors in Tehran last October.

according to Iran's Foundation of Martyrs and Veterans Affairs (FMVA), the chemical onslaught killed nearly 5000 Iranians and sickened more than 100,000. That doesn't include Iraqi victims: In March 1988, Iraq's forces attacked its own citizens with mustard and nerve agents in Halabja, killing as many as 5000 and wounding 7000.

Sulfur mustard, a family of compounds first used in World War I, left the deepest and most visible scars on survivors of the war. Three decades later, about 56,000 Iranians are coping with lingering health effects from the blistering agent, ranging from skin lesions and failing corneas to chronic obstructive lung disease and possibly cancer, says Tooba Ghazanfari, an immunologist at Shahed University here.

The scale of the atrocities means that Iran has a unique opportunity to study the long-term effects of chemical weapons. Ghazanfari is leading an effort at a dozen research centers across Iran to uncover how wartime mustard exposures wreaked molecular mayhem that, decades later, triggers illnesses and death. "I'm pretty amazed about the scale of exposures and how many people have been followed," says neuroscientist David Jett, director of the Countermeasures Against Chemical Threats (CounterACT) Program at the U.S. National Institutes of Health in Bethesda, Maryland, which is spearheading R&D on several promising methods of neutralizing such agents (see sidebar, p. 23).

The data, some of which are beginning to appear in Western journals, have unavoidable limitations. The Iranian researchers can only estimate the doses that victims absorbed, and complicating the picture is the fact that Iraqi forces sometimes attacked with mustard and nerve agents simultaneously. "That makes it really difficult to determine what's going on," says James Madsen, a physician and lead clinical consultant in USAMRICD's Chemical Casualty Care Division. Still, U.S. researchers say the Iranian findings are pointing to new molecular targets for treating mustard's long-term effects.

So far, Iran has not undertaken a similar study of nerve agent survivors, who also number in the thousands. Based on Japanese victims of sarin attacks by the Aum Shinrikyo doomsday cult in the mid-1990s and U.S. soldiers possibly exposed to sa-

rin in Iraq, researchers suspect that nerve agents leave a legacy of neurological damage. But chemical weapons experts have struggled to link sarin to specific chronic symptoms; they are hoping for some clarity from a U.S. review that is about to be released. And Iranian researchers say they may start their own probe of nerve agents' long-term health effects. "We want to get the knowledge out," says Mohammad-Reza Soroush, a physician here at the Janbazan Medical and Engineering Research Center.

can leave him gasping for breath. Lung infections land him for weeks at a stretch here at Sasan Hospital, Iran's premier facility for chemical weapons victims, which treats as many as 150 such patients on any given day. As Cherchi tells his life story in a hospital ward, his confident voice falters and, eyes widening, he reaches for an inhaler. "Chemical weapons are not fair," he says.

An old scourge ruined Cherchi's lungs. Called mustard because of its odor and the enormous yellowish blisters, or bullae, in a weapons factory on Ōkunoshima, an island in Japan, were exposed to mustard on the job. Studies flagged an elevated lung cancer risk, but failed to shed light on the mechanism of sulfur mustard's long-term effects, says pharmacologist Jeffrey Laskin, who directs a CounterACT center on chemical weapons countermeasures at Rutgers University's Robert Wood Johnson Medical School in Piscataway, New Jersey.

Iranian scientists are seeking answers. A decade ago, Ghazanfari and colleagues

> launched the Sardasht-Iran cohort study, named after a town in the northwest part of the country that was the site of one of the war's more reprehensible acts.

> On 28 June 1987, Iraqi aircraft dropped four 250-kilogram bombs filled with mustard on residential areas of Sardasht. "It was a completely innocent city," says Mohammad-Reza Vaez Mahdavi, a Shahed University physiologist who studies the survivors. Records show that 8025 of the 12,000-odd residents were exposed. A few dozen-mostly children and elderly-died within hours or days. About 1500 people suffered moderate to severe symptoms, and at least 100 have since succumbed to respiratory failure, Ghazanfari says.

> With funding from FMVA, Ghazanfari's team is tracking the health of 850 male victims, now aged 30 to 70. Most still reside in Sardasht. For a control

group, the scientists recruited 150 men of equivalent age in Rabat, a village 15 kilometers from Sardasht. During the war, Rabat was shelled often-but never with chemical weapons. "Both populations endured a lot of stress," Ghazanfari says. In principle, that allows her team to zero in on illnesses in Sardasht that can't be chalked up to stress or factors such as diet and economic status, which are similar in Sardasht and Rabat.

The Sardasht survivors have much higher rates of eye and skin disorders. A few have also developed cutaneous T-cell lymphoma at the site of old mustard burns. Emadi says the uncommon skin cancer may also explain mysterious lesions that doctors in the early 20th century noted in World War I mustard victims. But he says that benign skin lesions are far more common in the victims: patches that lack sweat glands or the ability to secrete sebum, resulting in intensely itchy, dry skin that's susceptible to infection.



Ali Reza Yazdanpanah holds up a photo of himself after he was exposed to sulfur mustard during the Iran-Iraq War. Now a docent at the Tehran Peace Museum, he calls the repeated use of chemical weapons against his nation "Iran's Hiroshima."

WITH HIS BEARISH FRAME and thick black beard, Ali Cherchi would be an imposing figure—if he weren't propped up in a hospital bed with an oxygen tube in his nose and an intravenous line plying him with antibiotics. His infection-riddled lungs are giving out.

Like many teenage boys filled with patriotic fervor during the war, Cherchi lied about his age, saying he was 18 instead of 17 so he could enlist in the army. One day in 1986, near the Persian Gulf in southern Iran, the Iraqis shelled his 200-strong platoon with mustard and tabun. Many of the young men had beards, so their gas masks did not fit tightly. "I felt like I was suffocating," Cherchi says. Only a few dozen survived the attack, and since then most of the rest have died. "I'm the champion for having lasted so long," he says with an ironic laugh.

For years, Cherchi indeed considered himself fortunate: He studied law, married, and had children. But now, a short stroll that it forms on the skin, bis(2-chloroethyl) sulfide-the primary member of a family of noxious compounds-debuted on the battlefield in July 1917, when Germany used it against U.K. and Canadian soldiers near Ypres, Belgium. After a mustard-filled artillery shell detonates, the chemical is dispersed as fine droplets that settle to the ground. In hot and dry climates, mustard can vaporize and is more likely to be inhaled. A mere 7 grams—a teaspoonful—is a lethal dose, Madsen says.

How the mustards produce their acute effects is well known. The oily liquid dissolves readily in fat, allowing it to penetrate skin. Inside cells, the chemical corrupts DNA and proteins. Recovery from the tissue damage can take months, and some World War I mustard survivors succumbed years later to respiratory failure.

Countries stockpiled sulfur mustard during World War II, but it saw limited use in battle. However, thousands of workers

How to defeat a nerve agent

ven 3 decades later, Seyed Naser Emadi's first encounter with nerve agents haunts him. In 1987, as a soldier fighting for Iran in its war with Iraq, he came across a hillside strewn with comrades killed by an Iraqi nerve agent, perhaps tabun or sarin. Unable to breathe, the victims had clawed at their necks to try to open a hole in their throats, recalls Emadi, now a dermatologist in Tehran. In fact, their windpipes were clear; the nerve agent had shut down control of breathing in the central nervous system. They "had no choice except death," he says.

The long-term effects of nerve agents remain uncertain (see main

story, p. 21), but with the right antidotes, these poisons need not be an immediate death sentence. A few years after Emadi's experience, U.S. soldiers in 1991's Gulf War carried autoinjectors filled with drugs that-in principlewould keep them breathing and protect them from seizures if Iraqi forces again unleashed nerve agents. They never did, most historians agree, but the threat remains real today, as chemical attacks in Syria's ongoing civil war make clear. It is spurring urgent efforts to find better countermeasures, with several promising compounds in the pipeline.

First synthesized by German chemists on the eve of World War II, nerve agents kill by binding to acetylcholinesterase (AChE), an enzyme that dismantles the neurotransmitter acetylcholine when it is released into synapses. One of the most efficient enzymes known, a single AChE molecule can hydrolyze 600,000 acetylcholine molecules per minute, says Palmer Taylor, a pharmacologist at the University of California, San Diego.

Nerve agents slot right into AChE's active center, a narrow gorge. Once one nestles there and forms a covalent bond with a serine group, AChE "can't spit it out," says James Madsen of the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) in Edgewood, Maryland. As acetylcholine builds up in the synapses, victims may develop chorea and dance ghoulishly before collapsing and writhing in seizures. Without medical intervention, the brain stops sending signals to the muscles that control respiration and maintain blood pressure: Victims drift into a coma and will probably stop breathing. Milligram amounts of nerve agents are enough to kill.

For defense against seizures, U.S. soldiers carry an anticonvulsant. Diazepam has been the treatment of choice, but the U.S. National Institutes of Health (NIH) in Bethesda, Maryland, and the U.S. Department of Defense have thrown their weight behind a faster-acting compound, midazolam. "We think it's going to be a much better drug in mass casualty situations." NIH neuroscientist David Jett says.

Other remedies take aim at the cause of the symptoms: excess acetylcholine. Atropine, a compound derived from the belladonna plant, blocks acetylcholine receptors and was used

to treat nerve agent victims in the Iran-Iraq War. But it can't prevent nerve agent molecules that do latch onto AChE from forming an irreversible bond with the enzyme, a process measured by the nerve agent's "aging" time. Sarin's aging time is about 5 hours. Soman, a less known but much-feared nerve agent, ages AChE in just 2 minutes.

Given only atropine, nerve agent survivors need weeks for their bodies to regenerate AChE. That's why victims are also given—as soon as possible after exposure—what Madsen calls the "true antidote" for nerve agent poisoning: oximes. Like a molecular crowbar, these chemicals jimmy the nerve agent off AChE before the complex ages.

In the United States, the only oxime approved for use against nerve agents is 2-pralidoxime chloride, or 2-PAM. But because

> 2-PAM is positively charged, little if any crosses the blood-brain barrier, Taylor says. That's a serious drawback, because nerve agents exert their most destructive effects in the brain.

In 2001, Taylor teamed up with K. Barry Sharpless, a chemist at the Scripps Research Institute in San Diego, to create oximes that lose protons to become a neutral species; the compounds were screened for those that, once inside the brain, can regain a positive charge, which is crucial for the molecular jimmying to work. Taylor's team has tested its front-runner in mice and in macaques exposed to sarin, and in August 2017 reported that it rapidly reversed symptoms.

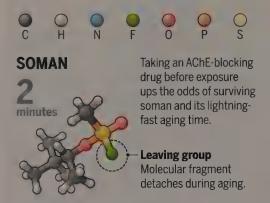
A group led by Carlos Valdez, a chemist at the Lawrence Livermore National Laboratory in Livermore, California, has also developed a potent neutral oxime that readily enters the brain of guinea pigs. And Janice Chambers, a biologist at Mississippi State University in Starkville, together with her late husband, Howard Chambers, created a novel oxime with a fat-loving group that should help it traverse the blood-brain barrier. It alleviated symptoms in rats exposed to nerve agent surrogates and is undergoing further testing.

The likeliest nerve agent to be used in future attacks, defense experts say, is sarin. It's as volatile as water, disperses widely in the air, and doesn't linger, so attacking troops could enter a battlefield soon after its release. But soman, too, is a formidable threat. It evaporates only a fifth as quickly as sarin and therefore persists longer in the environment. Soman's tactical value "would be to deny the use of key terrain" by contaminating it, says Jonathan Newmark, a neurologist formerly with USAMRICD.

Soman's lightning-fast aging time means that oximes won't work against it. During the Gulf War, soldiers deemed at a risk of exposure out in the field proactively took pyridostigmine bromide, a drug used to treat a neuromuscular disorder called myasthenia gravis. It lodges temporarily in the AChE gorge, thwarting nerve agent binding. A countermeasure that has to be taken before exposure has obvious limitations: If terrorists were to get their hands on soman, they would almost certainly give no warning. - Richard Stone

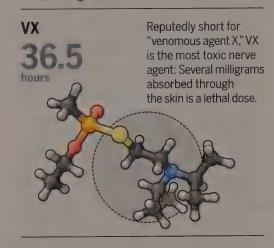
A deadly grasp

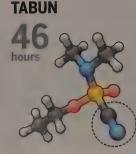
Nerve agents bind to acetylcholinesterase (AChE), an enzyme that controls levels of the neurotransmitter acetylcholine. Each agent's "aging time," listed here, indicates how long it takes for the bond to become irreversible.



SARIN

Responders would have a brief window after exposure to treat sarin victims with oxime, which pries the nerve agent off AChE before aging is complete.





A German chemist discovered tabun's lethality in humans by accident in 1936. The first nerve agent saw extensive use during the Iran-Iraq War.

IT'S IN THE LUNGS where mustard wreaks the most havoc. "Many soldiers on the battlefield don't have symptoms, but years later they come to us with problems," says Mostafa Ghanei, a physician at Baqiyatallah University of Medical Sciences here who consults for the Organisation for the Prohibition of Chemical Weapons (OPCW) in The Hague, Netherlands. Mustard creates what he calls a "unique pathology": peribronchial fibrosis-a thickening in tissues surrounding the bronchi-coupled with an airway obstruction called bronchiolitis obliterans.

To get at the roots of these symptoms, the Iranian team is probing a complex cascade of biochemical perturbations that persists in mustard survivors. They have detected, for example, revved up signaling molecules involved in inflammation, such as tumor necrosis factor α (TNF α) and other cytokines; skewed populations of natural killer cells and other immune cells; imbalances in the protein-destroying enzymes called proteases; and a shortening of the telomeres, the "end caps" on chromosomes, which indicates prematurely aged cells.

"They're doing a really good job documenting what's going on," Laskin says. But why the cellular dysfunction occurs decades after exposure is still a puzzle, he adds.

DNA methylation and other epigenetic alterations-chemical changes to DNA that can alter a gene's expression without affecting its protein-coding sequence-may underlie some of the lasting biochemical havoc, Ghanei says. He and colleagues have found evidence, for example, that methylation of tumor suppressor genes may inactivate them and help explain the development of peribronchial fibrosis as well as slightly elevated rates of certain cancers, in particular blood cancers.

In the past, Ghanei says, mustard victims with chronic symptoms were given standard treatments for chronic pulmonary disorders such as asthma: corticosteroids to tamp down inflammation and β2-agonists for smooth muscle relaxation. He and his colleagues now view oxidative stress as a prime target, because levels of an important antioxidant and an enzyme that scavenges destructive free radicals are suppressed in mustard victims. Iranian doctors now treat chronic mustard patients with antioxidants such as N-acetylcysteine, which alleviates the frequency and severity of bronchiolitis attacks, Ghanei has found.

Researchers in the United States are exploring other potential treatments. Drugs that neutralize TNFa might help, Laskin says. Another promising lead came when pediatric pulmonologist Livia Veress of the University of Colorado in Denver and colleagues zeroed in on fibrin clots in rats

Weapons in waiting

n the night of 26 October 2002. Russian special forces raided the Dubrovka Theater in Moscow, where terrorists were holding several hundred hostages. They had pumped a narcotic aerosol into the hall, aiming to incapacitate the terrorists. But the vapor was so potent that many hostages lapsed into a coma, and 124 of them died. In a nifty bit of detective work, the Defence Science and Technology Laboratory in Porton Down, U.K., analyzed clothing and urine from three survivors and revealed in 2012 that the narcotic was a combination of two fentanyl derivatives, carfentanil and remifentanil, both far more powerful than morphine.

The botched raid marked the debut of a new chemical weapon, one of many that worry experts. The U.S. Department of Homeland Security has a list of about 100 chemicals that pose a serious risk of being weaponized. Some were tested on animals decades ago, as the United States and other nations raced to acquire new chemical arms. Others are natural toxins that have long entranced weaponeers but are hard to disperse: tetrodotoxin from puffer fish, for example, and botulinum toxin, one of the most poisonous known compounds. (Sarin's lethal dose in mice is 100 micrograms per kilogram; botulinum toxin's is 0.001 micrograms per kilogram.) These days, the huge availability of synthetic opioids "is worrying a lot of folks," says David Jett, a neuroscientist at the U.S. National Institutes of Health (NIH) in Bethesda, Maryland.

The potential threats are spurring a search for new countermeasures. NIH is backing studies on whether opioid overdose therapies such as naloxone could be used if opioids are ever weaponized again. But Jett notes that "it's an arduous task to develop an antidote for every single threat agent." Instead, NIH has categorized chemicals of concern according to their toxidrome, or the broad set of symptoms they trigger. (Nerve agents, for example, have their own toxidrome, as do blistering agents such as sulfur mustard.) The aim, Jett says, is to develop antidotes "that work for most, if not all, chemicals in a toxidrome." -Richard Stone

exposed to mustard. When she dissected the animals, she pulled out of their lungs white clots called casts that looked like "tree branches," she says. "I realized I'd seen that before"-in children with plastic bronchitis, a rare complication of surgery to repair congenital heart defects. Veress tested a clot-busting drug, tissue plasminogen activator (tPA), in rats exposed to normally lethal mustard doses-they all survived. Her team has since given tPA to seven children with plastic bronchitis, which normally has a mortality rate of up to 60%. "These are really sick kids," she says. All survived; other hospitals have treated 22 children, all of whom recovered, too. "It's pretty darn cool," Veress says. Her team is working toward U.S. Food and Drug Administration (FDA) approval for tPA's use for plastic bronchitis and next month, they will start testing the drug in pigs to further demonstrate its potential as a sulfur mustard treatment.

Those prospects, although tantalizing, are still on the horizon, and mustard remains a grave concern: In March 2016, the Islamic State group shelled a village in Iraq near Kirkuk with rockets filled with mustard. "It is just as much of a threat now as it was in the Iran-Iraq War," Madsen says.

THAT IS EQUALLY TRUE for the nerve agents, which many nations still stockpile and which were used as recently as last April against civilians and rebel soldiers in Syria.

Syed Abbas Foroutan remembers their grim debut. In March 1984, he was a young army doctor running a unit for treating mustard victims when some soldiers with unusual symptoms were brought in. They were coughing and short of breath, and some were vomiting and drifting in and out of consciousness. It was clear to the doctors that they weren't dealing with mustard. "We could only say it was not this, not that," Foroutan says-until he found a symptom common to all the victims: "Their pupils were constricted to pinholes."

That pointed to a nerve agent. Such weapons form a covalent bond to and inactivate acetylcholinesterase (AChE), an enzyme that breaks down acetylcholine in synapses, thereby regulating levels of the neurotransmitter. A few days later, comatose victims started arriving to the clinic. Foroutan checked their AChE levels-they were near zero. The clincher was that the ill soldiers responded to atropine, which binds to and blocks acetylcholine receptors, reducing the effects of excess levels of the neurotransmitter. That realization was the beginning of a grave challenge for a young doctor fresh out of medical school, says Newmark, who has studied Foroutan's experiences. "He was the world's first physician to care for battlefield nerve agent casualties."

Undeterred by charges that it was engaging in a war crime, Iraq repeatedly shelled Iranian forces with tabun, sarin, and a binary form of the agent, cyclosarin. As Iraqi chemists learned to synthesize nerve agents with fewer contaminants and the military became more adroit at delivering them, the attacks were deadlier. Some casualties went "from fully functioning to seizing and comatose in seconds," Newmark says. Emadi, who witnessed such deaths, calls the victims "chemical martyrs."

To cope with the more potent threat, Foroutan pumped afflicted patients with massive doses of atropine-up to 10 times the amount that the United States and the North Atlantic Treaty Organization advise, near the threshold where the drug itself becomes toxic. "Foroutan was doing the best he could with what he had," Madsen says.

The radical treatment paid off, Newmark says. In most nerve agent fatalities, death occurs after the agent binds to enough AChE to block signal transmission from the brain's respiratory center to the diaphragm, paralyzing the muscle that's vital for breathing. "It takes fairly high doses of atropine to get into the brain," Madsen says. "What Foroutan did was intuitive. He gave so much atropine, we think that it started to take care of the [central nervous system] effects." Newmark puts it this way: "If a soldier got a snoot full of nerve agent, he was lucky to get to Foroutan's unit."

But what happened in later years to Foroutan's patients-and to thousands of others who survived nerve agent attacks—is largely unknown. That frustrates researchers, who would like to follow up on hints that exposure takes a long-term toll.

One clue comes from an episode in the United States, during Prohibition, when doctors started seeing patients with muscle spasms, tingling and numbness in the legs, and, in some cases, paralysis. The cause turned out to be an adulterant, triorthocresyl phosphate, in a bootleg ginger liquor. That compound, like nerve agents, is an organophosphate, and the symptoms that afflicted the "Ginger Jake" drinkers-some 30,000 documented cases-are now known as organophosphate-induced delayed neurotoxicity (OPIDN). The same syndrome affected one victim of the Aum Shinrikyo cult's sarin terrorism, says Bahie Abou-Donia, a neurobiologist at Duke University in Durham, North Carolina. "It would not surprise me to see OP-IDN in the Iranian survivors," Madsen says.

There's firmer evidence, he says, that nerve agents mess with the mind. Studies of the Aum Shinrikyo survivors link exposure "even at really low doses" to symptoms

such as nightmares, headaches, drowsiness, confusion, memory deficits, irritability, and depression-a syndrome called organophosphorus-ester-induced neurotoxicity (OPICN). In March 1991, during the Gulf War, tens of thousands of U.S. soldiers may have been exposed to low doses of sarin after the destruction of an Iraqi ammunition dump, says Abou-Donia, a sarin expert. An unknown percentage of cases of the still-controversial Gulf War Syndrome, he says, may in fact be OPICN.

need a Sardasht cohort for nerve cases," he says. It could even be an international effort, Newmark suggests, if Iran were to set up a collaboration under OPCW's auspices. "It's important to know if neuropathology and resultant long-term effects actually occur in humans," Jett adds, "before we approach the FDA for approval of a neuroprotectant drug."

In the heart of Tehran, a modest one-story building is a living memorial to Iran's chemical weapons victims. Inside the Tehran



Female volunteers learning to use gas masks in a Tehran mosque in May 1988. In the waning days of the war, Iraq resorted more frequently to bombarding soldiers and civilians with sulfur mustard and nerve agents.

More clarity on the long-term effects of nerve agents will come from a U.S. National Toxicology Program report on sarin. The draft review concludes that sarin's chronic effects include AChE inhibition, vision and memory problems in people, as well as nerve pathology detailed in animal studies.

The Iranian veterans could yield a more definitive picture. "Whatever comes out of Iran on this," Newmark says, "will be the best human data that exists." But so far, no one is gathering it. After the war, Foroutan became a professor—he's now at Shahid Beheshti University of Medical Sciences here-and has not tracked his former patients. And zeroing in on late nerve agent effects is not simple. "We don't have reliable information on who was exposed solely to nerve agents," says Janbazan medical researcher Batool Mousavi, because of the Iraqi practice of bombarding troops with nerve agents and mustard simultaneously or on consecutive days.

Foroutan believes that it's not too late to delve into the long-term consequences. "We

Peace Museum, exhibits recount the global history of chemical warfare. Chemical victims of the Iran-Iraq War volunteer as docents. One is named Ali Reza Yazdanpanah.

When he was 15 years old, Yazdanpanah tricked his mother into signing his enlistment papers. Several weeks later, he and 31 other young men in his regiment were exposed to mustard near Khorramshahr, a port city on the Persian Gulf, near the Iraq border. Some of the blistering agent got in his eyes. "I lost my sight for some time," he says. It came back, but he has had to endure four cornea transplantations, and is on a waiting list for a new pair of lungs. He says he has never felt completely well.

Yazdanpanah is keeping the memory alive. And as a subject in Iran's research efforts, he hopes his experience will benefit future victims of these ghoulish weapons. "The best years of my life," he says, "were stolen from me."

Reporting for these stories was supported by the Pulitzer Center on Crisis Reporting.



NEXTGEN VOICES

Research resolutions

We asked young scientists this question: What is your New Year's resolution for your field? Describe one thing that your field's research community could do better in the coming year. We received responses from scientists around the world representing a variety of fields. Excerpts of their responses are printed below. -Jennifer Sills

Communication and outreach

Environmental scientists should connect with people who depend on natural resources, such as farmers, fishermen, and hunters. This year, we should strive to start a dialogue, build trust, and ultimately create collaborative research projects for more sustainable use and management of natural resources.

Felicia Olmeta-Schult

Environmental and Natural Resource Sciences. Washington State University, Vancouver, WA 98686, USA. Email: folmeta@wsu.edu

In a world where fact checking is at an all-time low and the positive public perception of science is dwindling, scientists

must learn to effectively communicate. Plant pathologists are in an ideal position to address this issue-they are centered between farmers, scientists, and the public. This year, it is imperative that our field works toward meaningful and targeted collaboration.

Lauren Massa Segal

Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, NE 68583, USA. Email: Isegal3@gmail.com

Forensic statisticians need to do a better job of communicating with lawyers, judges, and other practitioners. Poor communication in the forensic and legal fields can lead to miscarriages of justice. It is our responsibility to work with practitioners to improve forensic science techniques and the discussion of such techniques in the courtroom.

Sam Tyner

Center for Statistics and Applications in Forensic Evidence, Iowa State University, Ames, IA 50014, USA. Email: sctyner@iastate.edu

Earth scientists should resolve to talk more publicly about what we do know. Traditionally, scientists emphasize uncertain results that require more research. But taking this tack with the public invites confusion. Instead, we should be talking about our well-established findings. For example, we are certain that Earth's ice is melting, with substantial consequences for coastal erosion and flooding, saltwater inundation, and water security. We are also certain that human action can postpone the arrival of the most dire effects.

Twila Alexandra Moon

National Snow and Ice Data Center, University of Colorado, Boulder, CO 80309, USA. Email: twila.science@gmail.com

In the current U.S. political climate. science is under attack. Our representatives circulate falsehoods that blatantly contradict scientific literature. This year, the medical research community's

A natural historian resolves to better explain the value of museum collections to the public.

engagement in politics must expand beyond matters pertaining to the NIH budget. We can begin by pressuring our representatives and making our voices heard. But to initiate the path toward systemic change, we must ultimately cultivate challengers of the political status quo from within our research communities. It is time to bring our passion from the lab bench to the podium.

Ryan Dz-Wei Chow

Yale School of Medicine, New Haven, CT 06510, USA. Email: ryan.chow@yale.edu

My New Year's resolution for biologists: Do a better job explaining to the public why natural history museum collections are important. Natural historians are sometimes misunderstood as hunters or stamp collectors, but their collections, when curated ethically and legally, can open up a world of knowledge about our planet.

Prosanta Chakrabarty

Museum of Natural Science, Louisiana State University, Baton Rouge, LA 70803, USA. Email: prosanta@lsu.edu

Deep sequencing and genome-editing technologies make personalized medicine, gene therapies, and transgenic livestock possible. Yet manipulation of genetic material carries a social stigma, due to ethical and ecological concerns. To overcome this stigma, the gene-editing community needs to better communicate the implications (good or bad) of our research to the public.

Martin Pacesa

Department of Biochemistry, University of Zurich, 8057 Zurich, Switzerland, Email: m.pacesa@bioc.uzh.ch

Interdisciplinary collaboration

In 2018, I hope that human genetics researchers will increase our understanding of the biological mechanisms underlying genome-wide association study data by collaborating with computational and experimental scientists.

Anna Igorevna Podgornaia

Merck Research Labs, Boston, MA 02115, USA. Email: anna.podgornaia@merck.com

When infectious diseases strike, serum from survivors can be administered to others to confer passive immunity. However, serum-based therapy is inefficient and expensive. Moving forward, immunologists and public health officials should strengthen their pipelines to identify such survivors and sequence their antibodies,

thereby enabling mass production of the protective antibodies.

Jennifer Chen

Yale School of Medicine, New Haven, CT 06510, USA. Email: jennifer.s.chen@yale.edu

I hope that computational biologists can take part in more multi-institutional projects that involve both computational and experimental researchers from academia and industry. Such collaboration could help us solve real-life practical problems.

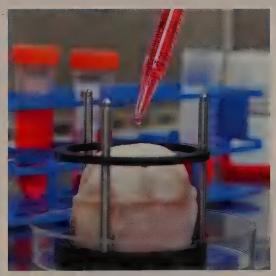
Bipin Singh

School of Engineering and Technology, BML Munjal University, Gurgaon, Haryana 122413, India. Email: bipin.singh@bmu.edu.in

For kidney disease researchers, interdisciplinary collaboration between scientists focusing on renal diseases. cell reprogramming, 3D printing, and biomechanics is urgently needed this year to push forward the development of artificial organs.

Ho Cao

Core Research Laboratory, The Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an Shaanxi, 710004, China. Email: bocao@vip.qq.com



A kidney researcher hopes that collaborations will lead to artificial kidneys similar to this bladder.

Neuromorphic engineers try to create circuits and algorithms that function like the human brain. However, once a basic biological model is implemented, most efforts focus on algorithmic efficiency, which makes the system less realistic. We need more input from neuroscientists. My New Year's resolution is to create a close-knit community of engineers and scientists.

Rishi Raj Singh Sidhu

Electrical Engineering, Centre of Excellence in IC Design (VIRTUS), Nanyang Technological University, Singapore, 639798, Singapore. Email: rishirajsidhu@gmail.com

Data sharing and analysis

In an era of next-generation sequencing and artificial intelligence processing of large amounts of biological data, the field of molecular biology should go beyond statistical power and fulfill three criteria: statistical, biological, and clinical significance. Often, statistically significant associations in the laboratory do not have significant clinical utility. We should adopt a clinical perspective that allows clinicians and scientists to harness the power of big data to improve the lives of patients.

Bryce W. Q. Tan

Department of Physiology, National University of Singapore, Singapore 117456, Singapore. Email: brycetan03@hotmail.com

The life sciences community should resolve to generate and interpret data more responsibly. We need to dispel myths and fears surrounding data analytics, adequately train our scientific workforce, and encourage open data and protocol sharing. We need to prevent data dredging, encourage complete and transparent reporting, avoid financial and nonfinancial conflicts, and reward reproducibility as much as novelty.

Prashant Sood

MRC Centre for Medical Mycology, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, UK. Email: drprashantsood@gmail.com

This year, geologists should emphasize observation-driven over model-driven science. Our models may speak to physically possible hypotheses, but they often fail to be predictive.

Stuart Parker

Idaho State University, Pocatello, ID 83204, USA. Email: parkstua@isu.edu

My New Year's resolution for psychology and neuroscience is to move beyond a "onesize-fits-all" approach to mental health treatments. We could improve treatment effectiveness by taking individual variability into account. In the next year, I hope that the field works to identify neurological, genetic, and psychological markers of who will best respond to specific interventions or combinations of interventions.

Matthew A. Scult

Department of Psychology and Neuroscience, Duke University, Durham, NC 27708, USA. Email: matthew.scult@duke.edu

In 2018, I think my field of cancer nanotechnology could benefit from more complete reporting of experimental details, particularly nanoparticle concentrations, animal experiment details, and cell experiment details. Most publications provide minimum explanation in these areas, which can make reproducing the findings difficult.

Desiree Van Haute

Jefferson, AR 72114, USA Email: desireevanhaute@gmail.com

My 2018 resolution would involve a community effort by biologists to integrate data from various sources in databases, which would become available to everyone as fast as possible. Analysis of these data by research labs with diverse interests would lead to an explosion of scientific discoveries.

Nikos Konstantinides

Department of Biology, New York University, New York, NY 10003, USA. Email: nk1845@nyu.edu

I wish that the field of education research could come together to (finally) agree upon a common core. Without agreement on the foundations of learning and teaching, the field remains divided and of limited use to practitioners and decision-makers.

Beat A. Schwendimann

Berkeley, CA 94720, USA Email: beat.schwendimann@gmail.com

Affordable research benefits

Arsenic in rice is a huge problem in Southeast Asia. My New Year's resolution for environmental scientists is to come up with low-cost and easily applicable solutions that allow local farmers to grow safe rice crops.

Sudhakar Srivastava

Institute of Environment and Sustainable Development, Banaras Hindu University, Varanasi, Uttar Pradesh 221005, India. Email: sudhakar.srivastava@gmail.com

At its current cost, there is no hope that next-generation sequencing will be accessible enough to affect medical diagnosis. Sequencing has become a profit-driven business. This year, biotechnology companies should take seriously the task of creating a \$100 genome, a cost directly controlled by reagent pricing. A lower price tag would make personalized medicine a global possibility, give better data sets for population studies, and make academic research more feasible.

Raffaele Fiorenza

Sequencing Operations Lab, New York Genome Center, New York, NY 10013, USA. Email: ralphie.fiorenza@gmail.com

This year, we need to discuss how we will achieve sustainable and affordable oncology with relevant stakeholders, from consumers, researchers, and clinicians to leaders in industry and government. Exploring new funding models and financial structures that continue to incentivize discovery and innovation will be critical for our continued success in treating latestage cancer.

Ken Dutton-Regester

QIMR Berghofer Medical Research Institute, Brisbane, QLD 4006, Australia. Email: ken.dutton-regester@qimrberghofer.edu.au

Diversity in science

My research resolution for ecology would be to ensure that those who have the chance to research biodiversity are as diverse as the communities we study. Ecologists need to do more to recognize the contributions of women and minority researchers and to break down systemic barriers to their

inclusion, such as the prevalence of unpaid "voluntary" field experience positions. We study behavior and species interactions in the field; let's be as aware of our own biases.

Rachel Hale

Ocean and Earth Science, National Oceanography Centre Southampton, University of Southampton Waterfront Campus, Southampton, Hampshire SO14 3ZH, UK. Email: r.hale@soton.ac.uk

In 2018, instead of focusing on postdoc positions, physics advisers at research institutions should customize career plans for their Ph.D. candidates that take into account each individual's goals, interests, and abilities.

Emre Ozan Polat

ICFO-The Institute of Photonic Sciences, 08860 Barcelona, Spain. Email: emre-ozan.polat@icfo.es

Postdoctoral fellows are a major driving force of biomedical research, but many postdocs are undercompensated and underrecognized. Our field should remedy this by acknowledging their role as intellectually independent scientists. More invitations could be directly extended to postdocs to pen commentaries, serve as panelists, review grant proposals, and referee manuscripts. Many postdocs already assist their lab heads in review tasks; inviting them to formally serve as reviewers will be a major step toward better postdoc recognition.

Edward Lau

School of Medicine, Stanford University, Palo Alto, CA 94305, USA. Email: lau1@stanford.edu

My New Year's resolution for ecologists: Pay more attention to the science produced by women and underrepresented minorities. Ensure diversity of keynote speakers, panels, journal editors, and awards. Insist upon field excursions and labs that are supportive and inclusive so that all trainees can thrive.

Audrey L. Mayer

SFRES, Michigan Technological University, Houghton, MI 49931, USA. Email: almayer@mtu.edu

Although biology graduate programs now have a balance of men and women, the leaky pipeline persists. One challenge to increasing diversity is ongoing sexual harassment on campus, at conferences, and during field work. Such a hostile environment prevents us from creating an inclusive community. Everyone, men in particular, must step up to combat sexual harassment.

Easton R. White

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EVOLUTION

The genomics of climate change

A study of yellow warblers identifies genomic regions involved in climate change adaptation

variation necessary for adaptation) of yel-

low warblers (see the photo) to climate

change and predict future population de-

clines. In doing so, they produce a powerful

tool for estimating genomic vulnerability to

climate change and locate candidate genes

that are key for climate change adaptation.

By Mark J. Fitzpatrick and Allan H. Edelsparre

uman-induced climate change is causing rapidly changing global temperatures and extreme fluctuations in precipitation. These changes force organisms to adapt and evolve or face extinction. Understanding and predicting the evolutionary responses to climate change is critical for preserving biodiversity, but predictions are challenging because they involve interactions between adaptive plasticity (such as altered breeding times) and evolved responses (such as increased metabolism). On page 83 of this issue. Bay et al. (1) combine high-resolution genomic sequencing with population trends and global climate predictions to estimate the adaptive potential (that is, the genetic

Yellow warblers are migratory birds with a wide distribution across North America. They occupy a rich set of habitats that range from marshes and forests to urbanized areas. They can be found in lowlands as well as at high altitudes, such as in the Rocky Mountains in the west and the Appalachian Mountains in the east. Given their wide distribution and general abundance, recent

declines of some populations have raised concerns that yellow warblers may be negatively affected by climate change (2).

Bay et al. initially obtained DNA samples

from 229 birds, representing 21 populations that span the yellow warbler breeding range. They used more than 100,000 nucleotide markers [single-nucleotide polymorphisms

(SNPs)] to characterize the statistical associations between current population-level genetic variation and 25 environmental variables associated with climate change. The variables included factors associated with precipitation, temperature, elevation, tree cover, and vegetation. The strongest associations between genomic and environmental variation were those related to precipitation. This makes sense, because changes in precipitation directly influence biomass, which in turn affects other factors, such as shelter and food availability. Extreme changes in precipitation remain among the most important, but least understood, consequences of rising temperatures across the globe (3).

By extracting information from their assembly of the yellow warbler genome, the authors assessed the adaptive potential of each population by piecing together a portrait of genomic regions that respond to climate-related changes and then searched for traces of selection in those regions. One

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major advantage to teasing out the adaptive potential from standing genetic variation is that it gives us the ability to ask whether such information can be used to forecast climate-related declines. By comparing the genomically determined adaptive potential of each population with local climate-related variables, Bay et al. were able to determine which populations have the greatest mismatch with their environment (as determined by the degree to which their genomic variation matches the environmental variation they experience), a metric they refer to as genomic vulnerability.

The most vulnerable populations largely reside along the Rocky Mountains, a region particularly affected by droughts over the past decade (4). The authors determined that these vulnerable populations coincide with recent population declines reported in the literature (5). This link between genomic vulnerability scores and realized population trends strongly suggests that genomic vulnerability accurately forecasts which popula-

"...behavioral genes may represent 'first responders' to climate change adaptation."

tions may be at risk of declining. This allows conservation efforts to target these identified areas and populations before environmental conditions become unsustainable.

The estimates of adaptive potential within species can be used to identify the traits and genes influenced by climate change. To do so, Bay et al. took a closer look within the important genomic regions discussed above. They found a strong association between climate (e.g., precipitation) and a SNP marker on chromosome 5, located very close to DRD4 and DEAF1; these two genes were previously linked with migration and exploratory behavior in several species, including birds (6) and humans (7). Initial responses to climate change likely involve highly plastic traits like behavior, where individuals themselves can act to better match their current environmental conditions; examples include avoidance behavior, retreating or reclaiming new habitats, diet modifications, dispersal, and migration.

Although the direct involvement of DRD4/DEAF1 needs to be established, their potential role in climate change adaptation is intriguing and raises the possibility that behavioral genes may represent "first responders" to climate change adaptation. If these adaptations involve dietary changes, dispersal, and migration, then additional candidate genes may include Pgi (8), npr-1 (9), and foraging (10), all of which have been associated with food search and dispersal.

Identifying general features of evolutionary responses to climate change is a crucial, but intricate, mission. Climate-driven adaptations will necessarily involve the genome, but time- and context-dependent factors will likely influence the specific pathway that any given population takes (including extinction). Some scenarios will directly lead to the evolution of new/modified alleles, whereas other scenarios may exist without any traditional genomic responses but instead be regulated by factors such as adaptive plasticity and epigenetics. The latter is particularly interesting, because both DRD4 (11) and foraging (12) can be regulated epigenetically to produce variations in behavioral tendencies that underlie food-searching and exploratory strategies within populations.

Climate change is happening and, despite growing global concern, is predicted to progress (13). Bay et al.'s study of the yellow warbler shows that, although populations can evolve in response to climate change, this response is limited by the adaptive potential and genomic vulnerability found among populations. A critical next step will be to determine how broadly these findings apply to other species and communities, especially those that are highly threatened. Time is of the essence. The widespread impact of climate change on our ecosystems and biodiversity has occurred over a relatively short time scale (about 50 years). According to the global forecasts of future climate change, the pervasive impact and strain on biodiversity will only intensify (3).

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CELL BIOLOGY

A new mitotic activity comes into focus

The ATR kinase has a role in mitosis to promote chromosome segregation

By Joshua C. Saldivar and Karlene A. Cimprich

uring mitosis, each duplicated chromosome must be accurately attached to the microtubule spindle, which pulls the chromosomes to opposite poles of the cell, where they are segregated to daughter cells. A number of mitotic kinases orchestrate mitosis to ensure accurate segregation, including cyclin-dependent kinase 1 (CDK1), the Pololike kinases, and the Aurora kinases (1). The kinase ATR (ataxia telangiectasia and Rad3related), which is involved in DNA damage responses during interphase of the cell cycle, has also been shown to prevent chromosome segregation errors (2). However, this role of ATR was presumed to be an indirect effect. On page 108 of this issue, Kabeche et al. (3) unveil a mitosis-specific ATR activity that ensures proper chromosome segregation and that this activity is dependent on a specific three-stranded nucleic acid structure known as an R loop.

ATR is a master regulator of the cellular responses to DNA damage and DNA replication stress by controlling cell-cycle progression, replication origin firing, replication fork stability, and DNA repair (4). These functions are mediated by the kinase activity of ATR and safeguard genome integrity. Kabeche et al. found that ATR is localized to and active at mitotic centromeres, the chromosomal region where the microtubule spindle attaches (see the figure). Because no mitosis-specific function of ATR was previously known, this is an intriguing observation.

Microtubules of the mitotic spindle attach to kinetochores at centromeres to facilitate chromosome segregation (5). Given the local activation of ATR at the centromeres of mitotic chromosomes, the authors assessed chromosome segregation

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when ATR was inactivated in mitosis and observed "lagging" chromosomes in anaphase (a stage of mitosis). A lagging chromosome is observed when it is not accurately attached to the mitotic spindle as the spindle pulls chromosomes to opposite spindle poles (see the figure). Failure to properly segregate chromosomes can lead to aneuploidy (an abnormal number of chromosomes in a cell) (5). Importantly, mitotic inactivation of ATR resulted in aneuploidy, suggesting that the mitosis-specific activity of ATR promotes accurate chromosome segregation.

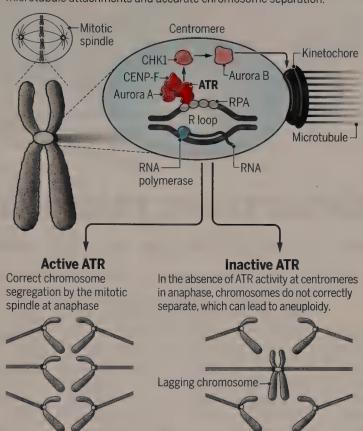
On the surface, this specific ATR activity appears counterproductive. Throughout interphase, ATR suppresses the activity of multiple CDKs, which controls DNA replication and cell-cycle progression. However, mitosis is a period of high CDK1 activity. It is unknown why ATR activation in mitosis does not suppress CDK1 activity and delay mitotic progression. One possibility is that the pathways that link the ATR pathway to CDK1 inactivation are rewired in mitosis. Alternatively, the focal activity of ATR at centromeres may spatially separate ATR and CDK1 throughout mitosis.

Kabeche et al. investigated how ATR is activated in mitosis at centromeres. In interphase, ATR is activated by specific DNA structures that form during DNA replication (S phase) or at DNA damage sites. Minimally, these structures consist of single-stranded DNA (ssDNA) and a 5'-ended ssDNA-double-stranded DNA junction (4). Because mitotic chromosomes are highly condensed and residual replication structures are processed by mitotic nucleases, it is unlikely that these structures would be abundant and specific to centromeres to activate ATR. Moreover, in S phase, centromeric repeat regions form structures that attenuate ATR activation (6).

Kabeche et al. speculated that R loops may be the centromere-specific ATR-activating structures. R loops are RNA-DNA hybrids with a displaced ssDNA loop [which is bound by replication protein A (RPA)] that could serve as a platform for ATR recruitment. Indeed, they showed that mitotic centromeres are enriched for RNA-DNA hybrids. Disruption of these hybrids prevented ATR recruitment and kinase activity at centromeres and increased the frequency of lagging chromosomes. The recruitment of ATR to centromeric R loops is complex because it depends on Aurora A and centromere protein F (CENP-F), which may bring ATR to the cen-

R loops activate ATR in mitosis

ATR activity at the centromere of mitotic chromosomes is induced by R loops and requires Aurora A and CENP-F. ATR phosphorylates CHK1, which phosphorylates Aurora B, which promotes kinetochore-microtubule attachments and accurate chromosome separation.



tromere. Once recruited, exactly how ATR is activated at centromeres remains unclear. In other contexts, ssDNA is only sufficient to recruit ATR to chromatin; activation requires a second factor to stimulate kinase activity. Understanding ATR activation at R loops will be an interesting area for future investigation.

How does centromeric ATR promote chromosome segregation? Inhibition of ATR kinase activity decreased Aurora B activity at centromeres, where Aurora B normally ensures that sister kinetochores are attached to microtubules from opposite spindle poles. Loss of this Aurora B function leads to lagging chromosomes (7), which is in agreement with a mitotic role of ATR to increase Aurora B activity. Kabeche *et al.* also showed that the main downstream effector of ATR, checkpoint kinase 1 (CHKI), is needed for full activation of Aurora B, which is consistent with a previous study (8).

One of the intriguing findings is the involvement of R loops in ATR-Aurora B signaling. R loops can be harmful and induce DNA breaks and genome instability (9), but they are also known to have important regulatory roles in transcription (10). Kabeche et al. show that in the context of mitotic centromeres, R loops serve as a signaling hub for the ATR pathway and are critical for chromosome stability. Whether other processes and signaling pathways intersect at and depend

on R loops remains to be explored.

How R loops form at centromeres during mitosis is also a mystery. Although most transcription is downregulated in mitosis, there is active transcription at centromeres (11, 12). Whether this transcription leads to R loop formation is unclear. In fact, previous work concluded that singlestranded long noncoding RNAs (Inc-RNAs) are formed and required for the centromeric localization of the chromosomal passenger complex, which includes Aurora B, potentiation of Aurora B kinase activity, and accurate chromosome segregation (12). The functional similarities of R loops and lncRNAs are striking and raise numerous questions, including whether these nucleic acids have distinct functions at centromeres or whether they are somehow related.

Kabeche *et al.* conclude that centromeric R loops serve as a focus for mitotic ATR signaling, promoting full activation of Aurora B and ensuring accurate chromosome segregation. This finding may prompt consideration of ATR as a peer of the classic mitotic kinases. Many aspects of this story are surprising, not least being why cells need such a complex

response involving transcription, R loop formation, ATR recruitment, and consequent CHK1 phosphorylation to induce a moderate increase in Aurora B activity. Perhaps it allows the spatiotemporal modulation of Aurora B, preventing excessive Aurora B activity away from centromeres? It will also be interesting to learn whether centromeric R loops signal to proteins other than ATR and whether centromeric ATR has other downstream targets. Undoubtedly, Kabeche *et al.* will motivate new investigations into the importance of ATR during mitosis.

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the destructive potential of these cells. A

series of coinhibitory molecules, called

immune checkpoints, expressed by anti-

gen presenting cells are responsible for

switching off T cell activation and termi-

nating the immune response. Although

this regulatory system is essential for a

measured immune response, and thus for

host homeostasis, expression of immune

checkpoint molecules by tumor cells leads

to inactivation of cytotoxic CD8+ T cells (a

type of effector T lymphocyte) and, as a re-

sult, evasion of the antitumor immune re-

sponse. Unleashing the power of adaptive

immune responses by targeting immune

checkpoints, such as the programmed cell

Precision medicine using microbiota

Intestinal microbiota influence cancer patient responses to immunotherapy

By Christian Jobin

ccumulating evidence indicates that dysregulation of microbiota-host interactions associates with various diseases, including inflammatory bowel diseases (IBDs), colorectal cancer, diabetes, and liver cirrhosis (1). Recently, research has generated paradigm shifts in concepts about the interactions between bacteria and cancer therapeutic drugs. For example, bacteria modulate the antitumor efficacy in preclinical models of various chemotherapies (2-4) and immunotherapeutic agents (5, 6). Conceptually, these findings suggest that bacteria-mediated interactions with the immune system are essential for optimal drug efficacy. However, there is limited information regarding the functional impact of the composition of the human microbiome and therapeutic outcomes in cancer patients. On pages 91, 97, and 104 of this issue, Routy et al. (7), Gopalakrishnan et al. (8), and Matson et al. (9), respectively, address this important issue and demonstrate that patients can be stratified into responders and nonresponders to immunotherapy on the basis of the composition of their intestinal microbiomes, suggesting that microbiota should be considered when assessing therapeutic intervention.

Effector T lymphocytes represent a critical branch of the adaptive immune response to antigens, and controlling the length and strength of this activation is necessary to preserve healthy tissues from

death protein 1 (PD-1)-PD-1 ligand 1 (PD-L1) axis has emerged as a promising approach for cancer therapy in solid tumors. However, patient responses to immune checkpoint therapies are heterogeneous and transient, a phenomenon related to limited immune cell infiltration of tumors and an immunosuppressive tumor microenvironment. Because microbiota have a pronounced modulatory effect on the immune system, they may enhance responses to immune checkpoint therapies. In an effort to identify microbes associated with treatment responsiveness, Gopalakrishnan et al. surveyed the oral and intestinal microbiota of patients with metastatic melanoma undergoing anti-PD-1 therapy. Interestingly, patients responding to this therapy had a high relative abundance of bacteria of the Faecalibacterium genus, whereas nonresponding patients displayed a high relative abundance of bacteria of the order Bacteroidales in their feces (indicating the presence of these bac-

teria in the intestines). They observed that

the strongest fecal microbial predictors of

anti-PD-1 therapy response were bacterial diversity and abundance of Faecalibacterium and Bacteroidales. No such microbial correlations were observed in the oral cavity, suggesting that the intestinal community is the source of bacterial-immune synergy for response to anti-PD-1 therapy. By also analyzing patients with metastatic melanoma undergoing this therapy, Matson et al. found responding patients had an increased abundance of eight microbial species, including Bifidobacterium longum. The presence of this species in the intestines of tumor-bearing mice was previously found to improve anti-PD-L1 therapy (6). Interestingly, two species were also associated with nonresponsiveness (Ruminococcus obeum and Roseburia intestinalis).

Routy et al. studied interactions between microbiota and response to anti-PD-1 treatment in patients with non-small cell lung cancer, renal cell carcinoma, and urothelial carcinoma. They observed that antibiotic exposure, taken during the course of cancer therapy to treat various infections, negatively correlates with patients' response to anti-PD-1 treatment. This suggests that disruption of microbial networks and loss of specific bacterial clades interfere with the efficacy of immune checkpoint blockade. Comparing the fecal microbiota of responders to nonresponders revealed increased relative abundance of Akkermansia muciniphila in patients showing favorable outcomes to anti-PD-1 treatment. Routy et al. surmised that microbial diversity and composition are a predictor of anti-PD-1 treatment response for these types of cancer. If the microbiota of responding patients containing immunoregulatory bacteria (for example, Akkermansia, Faecalibacterium. and Bifidobacterium) functionally drive

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anti-PD-1 efficacy, then it is expected that germ-free mice implanted with human tumor cells and transplanted with feces from these patients [a technique known as fecal microbiota transplantation (FMT)] would display better responses to treatment. Routy et al. and Gopalakrishnan et al. observed an improved response to anti-PD-1 therapy in mice receiving FMT from responsive patients compared to that in mice colonized with feces from nonresponsive patients. Gopalakrishnan et al. showed this improved response correlated with a higher abundance of Faecalibacterium in the feces of the mice. Matson et al. observed that anti-PD-L1 treatment was only effective in mice receiving FMT from responding patients, with five out of the eight strains originally found to be associated with anti-PD-1 response in patients detected in the

feces of the mice, including Bifidobacterium. In all three studies, tumors of mice receiving FMT from responsive patients displayed a higher density of CD8+ T cells compared to that in tumors in mice that received nonresponder FMT. This suggests that immune checkpoint therapy targets the repertoire and activity of host immune cells and induces antitumor responses mediated both by CD8+ T cells and a decrease in CD4+ regulatory T (T_{reg}) cells, which are immunosuppressive.

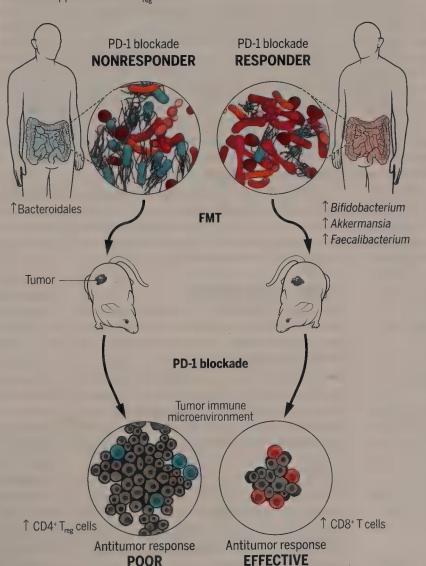
An important and clinically relevant issue is whether manipulation of the intestinal microbiome could turn patients that are nonresponsive to immune checkpoint blockade into responders. Routy et al. found that introduction of A. muciniphila to mice receiving human nonresponder FMT reversed the low response to PD-1 blockade, improving antitumor immune cell infiltration and activity in tumors. Overall, these studies report a fascinating interaction between intestinal bacteria and antitumor efficacy of PD-1 blockade in patients, suggesting that precision medicine strategies should include the intestinal microbiota as a potential treatment modifier (see the figure).

What is the evidence that microbiota-derived research could translate to new therapeutics? The most celebrated medical success targeting microbiota

came from the field of infectious diseases, with FMT-mediated treatment of recurrent Clostridium difficile infection (CDI), a leading cause of antibiotic-associated diarrhea with an alarming increased incidence in Europe, Asia, and the United States. Patients with recurrent CDI showed a 90% clinical response rate after FMT with feces from healthy donors (10). This resulted in considerable effort from both academic and private enterprises to design synthetic microbial communities to treat various microbiota-associated diseases such as CDI and IBDs. A similar route could be envisioned for cancer treatment, where synthetic microbial communities could be assembled to optimize patient responses to immunotherapy. Intestinal microbial communities have the capacity to modulate health status by engaging various immune and nonim-

The intestinal microbiota influences the efficacy of PD-1 blockade

The enrichment of specific microbial taxa in intestines correlates with response to PD-1 blockade in cancer patients. FMT from responders into tumor-bearing mice improved responses to anti-PD-1 therapy and correlated with increased antitumor CD8+T cells in the tumors. Mice receiving FMT from nonresponders did not respond to anti-PD-1 therapy, and tumors had a high density of immunosuppressive CD4+ T_{reg} cells.



mune cell types through microbial-derived structures (RNA, DNA, and membrane components) and by generating an impressive network of metabolites. In addition to having individualized microbiota, patients responding to immune checkpoint therapy could be predisposed to intestinal bacterial translocation into secondary lymphoid organs, where they could mount a specific antitumor immune response to allow better synergy with treatment. Untangling components that regulate the complex interaction between microbiota and host could also lead to the generation of targeted therapy, rather than nonspecific FMT.

Although these studies collectively agreed on the critical role of bacteria in defining responsiveness to anti-PD-1 therapy, no universal bacterial species define this response. The divergence in microbial

communities associated with the same therapeutic agent (anti-PD-1) may be related to the type of cancer (for example, metastatic melanoma with Faecalibacterium and non-small cell lung cancer, renal cell carcinoma, and urothelial carcinoma with Akkermansia) or patient population (U.S. versus European cohorts). However, both Matson et al. and Gopalakrishnan et al. conducted their studies using U.S.-based patient populations with metastatic melanoma, yet observed different PD-1 immunoregulatory bacterial species. The reason for this microorganism specificity is unclear, but it is likely that a convergent mechanism, which is yet to be identified, unifies them. Detailed mechanistic studies into how bacteria reenergize tumor immune microenvironments will be necessary to fully comprehend this phenomenon. Another intriguing observation is that strains of Akkermansia, Faecalibacterium, and Bifidobacterium have been associated with anti-inflammatory responses, a regulatory arm of the immune system that aims to prevent overactivation of the immune response and restores host homeostasis. For example, decreased relative abundance of A. muciniphila in the intestine associates with many diseases, including IBDs, type 2 diabetes, and liver diseases (11). Similarly, Faecalibacte-

rium prausnitzii down-regulates intestinal inflammation, which is associated with the production of specific metabolites, such as butyrate and salicylic acid derived from host cells or bacteria in the intestine and peripheral blood (12). Clearly, molecular characterization of intestinal Akkermansia, Faecalibacterium, and Bifidobacterium strains from cancer patients is needed to fully understand how they influence the tumor microenvironment and synergize with immune checkpoint blockade. These studies could lead to the isolation and characterization of microbial components that are responsible for the beneficial effects. For example, administration of Amuc_1100, a protein isolated from the outer membrane of A. muciniphila, reproduces the beneficial effect of the bacterium on diabetes in preclinical models (13).

The relationship between microbial communities and antitumor drug responses are complex. On the one hand, depletion of selective bacterial taxa by means of antibiotic exposure or other stressor conditions may diminish immunotherapy responses. On the other hand, the presence of specific microorganisms in local or distant sites may interfere with treatment through metabolic activities (14). For example, bacteria of the Enterobacteriaceae family, such as *Escherichia coli* strains, decrease efficacy of the chemotherapeutic agent gemcitabine by metabolizing and deactivating the active form of the drug, thereby negatively interfering with tumor response (15). Therefore, the presence of specific strains of bacteria may be able to modulate cancer progression and therapeutics, raising the possibility that precision medicine directed at the microbiota could inform physicians about prognosis and therapy. One could view the microbiota as a treasure trove for next-generation medicine, and tapping into this network may produce new therapeutic insights.

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OCEANS

Mind the seafloor

Research and regulations must be integrated to protect seafloor biota from future mining impacts

By Antie Boetius^{1,2,3} and Matthias Haeckel⁴

s human use of rare metals has diversified and risen with global development, metal ore deposits from the deep ocean floor are increasingly seen as an attractive future resource. Japan recently completed the first successful test for zinc extraction from the deep seabed. and the number of seafloor exploration licenses filed at the International Seabed Authority (ISA) has tripled in the past 5 years. Seafloor-mining equipment is being tested, and industrial-scale production in national waters could start in a few years. We call for integrated scientific studies of global metal resources, the fluxes and fates of metal uses,

"To be economical, any single operation would have to mine several hundred square kilometers of deep seafloor per year..."

and the ecological footprints of mining on land and in the sea, to critically assess the risks of deep-sea mining and the chances for alternative technologies. Given the increasing scientific evidence for long-lasting impacts of mining on the abyssal environment, precautionary regulations for commercial deep-sea mining are essential to protect marine ecosystems and their biodiversity.

REMOTE AND UNKNOWN

The seabed covers 70% of Earth's surface and is home to a virtually uncharted diversity of marine life. The ocean floor, at an average depth of 4 km, is characterized by pressures of several hundred bars, temperatures around the freezing point of water, and no sunlight for photosynthetic productivity. For humans, this environment is inhabitable, barely accessible, and extreme.

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et, the relatively stable environmental conditions of the deep sea have promoted a vast biodiversity of taxa that are not found in shallow waters or on land.

Because of its remoteness and the technical challenges associated with reaching the deep seabed, much less than one-thousandth of its area has been studied (1). As a result, little is known about how much deepsea species contributed to the evolution of life and biodiversity on Earth, how they can tap into unusual energy sources, how the sparse populations maintain enough standing stock in this vast realm, and how they adapt to perturbations. No country sustains the necessary amount of deep-sea observations required to answer such questions. The lack of long-term ecological time series is particularly problematic, making it difficult to decipher natural dynamics and anthropogenic perturbations (2). Recent findings suggest a tight coupling between the dynamics of surface and deep-sea processes and confirm that effects of climate change and pollution propagate quickly. But little is known about the resilience and recovery of deep-sea communities in the context of activities such as mining (3).

HAZARDS OF DEEP-SEA MINING

Critical metal resources in the deep sea include arsenic, copper, cobalt, nickel, lithium, platinum, tellurium, zinc, and many rare earth elements (4). The exploitable resources of the potentially prolific, 40-million-km² deep-sea area (5) could be worth \$20 trillion to \$30 trillion, considering current metal prices. However, these resources are not renewable, because polymetallic nodules and crusts grow very slowly over millions of years. Furthermore, mining is highly invasive, damaging the surface seafloor and entailing considerable risks for deep-sea life (6, 7). Even in shallow seas, there is no efficient governance for monitoring, managing, and protecting the oceans. The extensive seafloor damage caused by benthic trawling or by accidents such as the 2010 Deepwater Horizon oil spill in the Gulf of Mexico shows that ecological impacts on the deep sea remain literally out of sight in ocean resource management. Even in national waters, no system is in place to repair, restore, and compensate for loss of seafloor habitat.

On land, metal mining is highly destructive to the environment and can put the health of miners and local communities at risk, if not carefully regulated and managed. Mitigation actions such as habitat compensation, restoration, and adaptive management are expensive, but can minimize the damage to biodiversity and help to restore terrestrial landscapes within a few decades. However, it remains questionable whether any landbased mitigation mechanisms can serve as a blueprint for regulations of deep-sea mining (8). The high economic costs of ecological impact on land (9) are often used as an argument for deep-sea mining. Yet, such a "not-in-my-backyard" drive toward seafloor uses would violate the internationally affirmed precautionary approach, as reflected in principle 15 of the 1992 Rio Declaration on Environment and Development.

The ISA, which is responsible for controlling the exploitation of mineral resources in international waters, adopts this principle in its draft regulation, published on 8 August 2017 (10). However, concrete environmental objectives for protecting and conserving the deep-sea environment, including its biological diversity and ecological integrity, have not been agreed upon yet. To be economical, any single operation would have to mine several hundred square kilometers of deep seafloor per year, but this estimate does not include the costs of environmental management. At the anticipated scale of seafloor damage, monitoring, compensation, and restoration techniques would be extremely

expensive to implement. But without such measures, the use of deep-sea mineral resources to sustain further global economic growth would endanger the deep sea's genetic resources, which are a long-term target for biotechnology and medicine (II).

THE RESEARCH BASIS

Several countries are currently funding a range of activities connected to deep-sea mining, from developing technologies for deepsea raw materials extraction to researching the ecological consequences of deep-sea mining, but there is no central synthesis platform to bring together arising knowledge.

The MiningImpact project (a part of the Joint Programming Initiative Healthy and Productive Seas and Oceans) has brought together 11 European countries to study the





(Top) Holothurian in a polymetallic nodule field of the Clarion-Clipperton Fracture Zone of the Pacific Ocean. (Bottom) ROV Kiel 6000 push coring disturbed surface sediment of the 26-year old plow track of the DISCOL experiment in the Pacific Peru Basin.

environmental impacts of seafloor mining, with a focus on polymetallic nodules. The researchers revisited benthic impact experiments in the Pacific Peru Basin and in ISA contract areas in the Clarion-Clipperton Fracture Zone in the Pacific Ocean (see the photos). Some of these experiments were initiated up to 40 years ago and have now been studied with state-of-the-art methods (12). Key conclusions were that deep-sea ecosystems associated with polymetallic resources support a diverse fauna with high spatial and temporal variability and largely unknown connectivity; that the loss of seafloor integrity by mining reduces population densities and ecosystem functions for many decades; and that sediment plumes will likely blanket the seafloor up to several tens of kilometers outside the mined area

(13). On the basis of current scientific knowledge, the long-term risks of industrial-scale deep-sea mining to the marine environment seem unmanageable from both the economical and the ecological perspective.

However, predicting impacts on the basis of small-scale benthic impact experiments is associated with many uncertainties. Benthic trawling provides a rough analog for large-scale impact on seafloor integrity. In shelf seas, recovery of seafloor communities from benthic trawling can take less than a decade depending on the substrate (14), but decades to centuries are needed in deepwater habitats (15). In 2016, the European Union banned seabed trawling on continental slopes below 800 m to reduce risks for deep-sea life.

Dark, cold, energy-poor deepsea ecosystems are particularly vulnerable to mechanical disruption of the surface seafloor, which contains most of the food and microbial communities on which benthic fauna depend. Biogeochemical investigations as part of the MiningImpact experiments confirm that even the soft sediment seafloor would take many decades to hundreds of years to recover from the disturbance caused by nodule removal (13). The nodules and crusts themselves, which provide habitats to many deep-sea species, would need millions of years to grow back (4).

CAREFUL OCEAN GOVERNANCE

Strict environmental regulations

need to be formulated by the ISA as they finalize their regulations (10). Before any industrial activities could begin, long-term studies with realistic analogs to mining technologies would be needed. Conservation areas must closely match ecosystem characteristics of mined areas to safeguard abyssal biodiversity. Technologies for baseline studies and monitoring need to be standardized and regularly revised to reflect state-of-the-art science. Indicator sets for deep-sea ecosystem status and threshold values for harmful effects must be defined. Environmental management plans need to address uncertainties of sediment-plume dispersal. Finally, transparent and independent international scientific assessment of environmental management plans needs to be in place before any deep-sea mining

operations can start. Transparency also needs to be increased in the ISA's decisionmaking process for issuing contracts, as has become obvious by the latest approval of a 10,000-km² claim at the Mid-Atlantic Ridge (16) that includes the hydrothermal-vent systems of Lost City, Trans-Atlantic Geotraverse, and Broken Spur, which are key scientific research sites.

Before taking any risk to destroy deepsea habitats and endanger marine species, metal resources on land could be fully mapped and explored. Technological and social innovations could improve the way metals are used and recycled, and international politics could foster metal-market stability. In all of this, holistic science projects and stakeholder dialogue could help in finding solutions to the development of metal resources, uses, and fates. This would also provide the necessary time to valorize ocean life and its genetic resources (11). A new kind of international deep-sea science and policy, in which knowledge and governance of mineral and genetic resources as well as other ocean ecosystem services are integrated and channeled into international policy, would allow humanity to sustain the full range of options for the deep sea.

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IMMUNOLOGY

Lung inflammation originating in the gut

Parasite infection in the intestine can lead to inflammatory immune cells in the lung

By Jenny Mjösberg^{1,2} and Anna Rao¹

nnate lymphoid cells (ILCs) are a type of immune cell that are considered to be tissue-resident gatekeepers situated in mucosal membranes, where they contribute to both homeostasis and pathology (1). In healthy individuals, ILCs are involved in tissue repair, but ILCs have also been shown to participate in several types of inflammation, including allergy and asthma. Whereas ILCs can be found at low frequencies in the blood circulation, mucosal barriers such as the intestine and airways are enriched for ILCs (1). However, whether ILCs are in fact tissue resident in the sense that they selfrenew without substantial replenishment from other organs has been a topic of debate. The mechanisms of ILC circulation are important for understanding various

"...ozanimod and related drugs might interfere with the ILC gut-lung axis and be beneficial in chronic lung inflammation..."

types of inflammatory conditions and how they can be treated. On page 114 of this issue, Huang et al. (2) demonstrate that ILC2s are not obligate tissue-resident cells because they can be recruited from the gut to the lung and other organs in response to inflammatory signaling.

ILC2s contribute to so-called "type 2 immunity" in which they are typically activated by the cytokines interleukin-25 (IL-25) and IL-33 to produce large amounts of IL-5 and IL-13, which are key players in this type of inflammation. In 2015, Huang et al. (3) described a population of lung ILC2s characterized by high expression of killer cell lectin-like receptor subfamily G member 1

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(KLRG1) and the IL-25 receptor, IL-17 receptor-β (IL-17Rβ), but low expression of the IL-33 receptor, ST2 (KLRG1high ST2low ILC2s). In contrast to natural ILC2s (nILC2s), which reside in the lungs during homeostatic conditions, KLRG1high ST2low ILC2s were termed inflammatory ILC2s (iILC2s) as they arise in the lungs only after type 2 immunity induced by IL-25 exposure or gastrointestinal infection with the parasitic worm Nippostrongylus brasiliensis. Now Huang et al. (2) extend their observations using a model to study circulation of blood-borne cells between two surgically connected (parabiotic) mice to show that iILC2s accumulate in the lungs during these conditions because they are rapidly recruited within 3 to 5 days. These data challenge the existing view established following the seminal paper by Gasteiger et al. (4), who did not observe replenishment of ILC2s until day 15 after N. brasiliensis infection in parabiotic mice. The discrepancy in timing of ILC2 recruitment between these two studies might be due to different conditions in which mice were housed. Gasteiger et al. kept their mice on antibiotic treatment, whereas Huang et al. (2) did not. It is therefore possible that the intestinal microbiota might play an important role in ILC2 circulation.

Huang et al. (2) identify the intestine as the critical origin of iILC2s recruited to the lung upon IL-25 administration, revealing a striking ILC2 gut-lung recruitment axis (see the figure). Supporting the intestine as the major source of iILC2s in the lung, transcriptional analysis demonstrates that lung iILC2s are more similar to small intestine ILC2s (siILC2s) than to lung nILC2s.

The recruitment of iILC2s in the lung is a transient phenomenon. Twelve days after IL-25 administration, iILC2s are undetectable in the lung. However, parabiosis experiments reveal that 10% of nILC2s in the lung and 40% of ILC2s in the intestine originate from the donor mouse, suggesting that lung iILC2s can return to the intestine or display plasticity and convert into nILC2-like cells in the lung (see the figure). Because these nILC2-like cells in the lung are derived from activated iILC2s, it is possible that they make up tissue-resident epigenetically poised "memory" ILC2s, as previously reported in mice exposed to allergens (5). Memory ILC2s would enable a rapid and more efficient immune response following a repeated immune challenge. In addition to developing into nILC2-like cells, iILC2s express IL17A transcripts (2), indicative of transdifferentiation to ILC3s. It is interesting to note that nILC2s, which expand locally in the lung, express receptors for IL-1β and IL-18, raising the possibility that nILC2s are prone to transdifferentiate to ILC1s, as occurs in human chronic obstructive pulmonary disease (COPD) (6, 7). Hence, iILC2s and nILC2s serve distinct and important purposes in the lung: iILC2s

are recruited from the intestine for rapid and transient type 2 immunity, whereas nILC2s are largely tissue resident and potentially give rise to immunity against viral infections, for example.

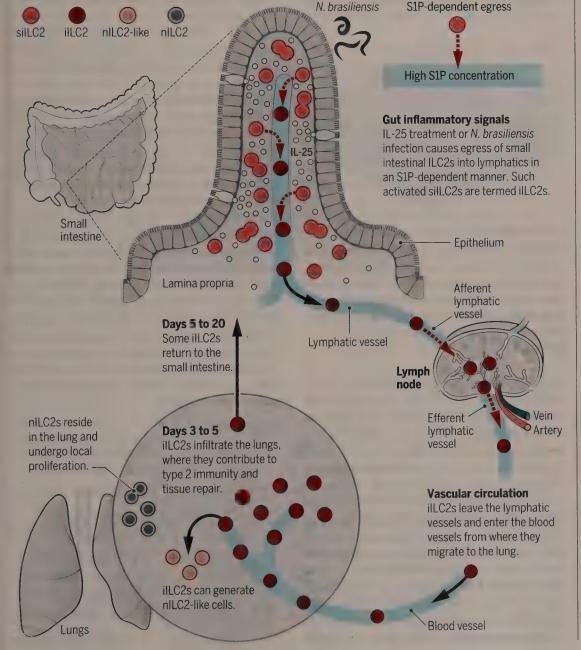
Huang et al. also describe the mechanism of IL-25-induced gut-lung recruitment. iILC2s are found in lymphatic vessels in the gut as well as in peripheral blood, suggesting that they circulate via the lymphatic system into the blood. In support of this, IL-25-activated iILC2s in the lung express sphingosine 1-phosphate (S1P) receptors S1P1 and S1P4 (2), indicating that iILC2s can cross lymphatic endothelial barriers, similar to T cells (8). Furthermore, like tissue-resident T cells (9), siILC2s in healthy mice lack expression of S1P1, reflecting their inability to egress from the gut in the absence of immune activation. Blocking S1P signaling with an inhibitor dramatically reduced the frequency of iILC2s in the lung and concordantly reduced the survival of mice following N. brasiliensis infection (2). As Gasteiger et al. observed a delay in the recruitment of ILC2s to the lung following N. brasiliensis infection in antibiotictreated mice (4), it is possible that the gut microbiota regulates SIP receptor expression on ILC2s. This, however, is unexplored.

Importantly, Huang et al. (2) show that S1P receptor-mediated egress is a conserved phenomenon that occurs both in innate (for example, ILCs) and adaptive (for example, T cells) lymphoid cells. However, it remains unclear if and by which mechanisms other ILC populations circulate during inflammation. It is also unknown how intestinal ILC2s enter the lung tissue from the blood. In addition to recruitment of activated ILC2s, as described by Huang et al. (2), it was recently shown that circulating human blood ILC precursors can seed tissues and differentiate to form all known ILC subtypes (10). However, it remains unclear how much recruitment of circulating precursors and local ILC generation contribute to the total ILC pool in an organ, relative to local proliferation (as observed for nILC2s) and plasticity and recruitment of differentiated ILCs (as seen for iILC2s) (2).

The ILC2 gut-lung axis identified by Huang et al. (2) is interesting in the context of the link between disturbances in the intestinal microbiota and lung diseases such as asthma and COPD (11). Considering that the S1P1 inhibitor ozanimod is currently in clinical trials for treatment of multiple sclerosis (12) and inflammatory bowel disease (13), perhaps ozanimod and related drugs might interfere with the ILC gut-lung axis and be beneficial in chronic lung inflammation, such as asthma and COPD.

Model for ILC2 gut-lung circulation

ILCs are involved in tissue repair, but ILCs have also been shown to participate in several types of inflammation, including allergy and asthma. Whereas ILCs can be found at low frequencies in the blood circulation, mucosal barriers such as the intestine and airways are enriched for ILCs. Huang et al. (2) demonstrate that ILC2s can be recruited from the gut to the lung in response to inflammatory signals.



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TRIBOLOGY

The contact sport of rough surfaces

Approximate models of interacting surfaces competed against a supercomputer solution

By Robert W. Carpick

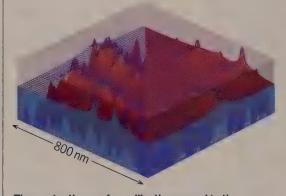
escribing the way two surfaces touch and make contact may seem simple, but it is not. Fully describing the elastic deformation of ideally smooth contacting bodies, under even low applied pressure, involves second-order partial differential equations and fourthrank elastic constant tensors. For more realistic rough surfaces, the problem becomes a multiscale exercise in surface-height statistics, even before including complex phenomena such as adhesion, plasticity, and fracture. A recent research competition, the "Contact Mechanics Challenge" (1), was designed to test various approximate methods for solving this problem. A hypothetical rough surface was generated, and the community was invited to model contact with this surface with competing theories for the calculation of properties, including contact area and pressure. A supercomputer-generated numerical solution was kept secret until competition entries were received. The comparison of results (2) provides insights into the relative merits of competing models and even experimental approaches to the problem.

Understanding contact and friction fascinated da Vinci, Coulomb, Prandtl, and de Gennes, among others (3). In 1881, Hertz solved the problem of two smooth, elastic spheres coming into contact, launching the field of "contact mechanics" (4) and leading to the development of equations for contact area, contact pressure, stresses, strains, and deformations for smooth, loaded contact geometries. However, real surfaces are rarely smooth. Even finely polished metals retain peaks and valleys with wavelengths ranging from nanometers to micrometers, leading to a seemingly random distribution of asperity contacts separated by stress-free noncontact zones (see the figure). Solving such contact problems is fundamental to understanding how gears turn, tires roll, and geckos climb (5), as well as for engineering any device with moving or contacting parts. It also underpins theories of friction, adhesion, lubrication, and wear, which can be empirical and highly system-specific.

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Just over 50 years ago, an elegant analysis of contacting rough surfaces assumed that surface summit heights, all with the same radii, had an approximately Gaussian distribution (6). This Greenwood-Williamson (GW) model is the most common approach to modeling rough surfaces and has since spawned a plethora of other models. These approaches involve both analytical extensions to the GW model as well as numerical approaches, with different degrees of complexity that account for effects such as plastic flow, adhesive forces, and anisotropic properties.

This wide variety of models not only makes it difficult for experimentalists and engineers to decide which approach to adopt but also throws into question their accuracy. Predictions from different models disagree strongly (as do their authors), and



The contacting surfaces like those used in the competition. The blue grid shows the deformations of the lowermost surface of an elastic solid (the left half is rendered more transparent) pressed against a rigid substrate with fractal roughness. The color range of the lower surface corresponds to height, enlarged ~30 times versus the in-plane directions. [Adapted from (10).]

experimental validations are scant. In late 2015 (I), Müser formulated a contact problem and, along with Spencer and Tysoe, invited researchers to partake in the Contact Mechanics Challenge. Müser used a supercomputer to conduct brute-force numerical simulations of a rough surface contacting a flat plate with a moderate amount of adhesion. The topography and material properties were shared on the internet, but the solution was held secret. The scientific community was challenged to share results from their preferred models for properties including the total contact area, the contact spot distribution, stresses, and deformations.

The response was impressive: 12 groups

involving more than 30 authors from Austria, England, France, Germany, Italy, Iran, the Netherlands, Taiwan, and the United States submitted entries that ranged from analytical solutions based on GW-inspired models to more recent fractal-based theories (7) and numerical solutions. One group even three-dimensionally printed a model of the rough surface and measured the resulting contact properties with a frustrated totalinternal-reflection method (8).

Müser and Greenwood compared and summarized the results (2). The multiscale approaches had the winning score, with an honorable mention for the experimental method, which matched the solution extremely well. Multiscale approaches based on Persson's theory (7) fitted much better than those based on GW for two reasons. First, ignoring small-scale roughness neglects high-pressure zones, whereas ignoring largescale roughness neglects substantial overall deformation as surfaces conform together. Second, with elastic solids, pushing down on one asperity creates a long-range elastic field that affects the neighbors by pushing them down as well. This effect renders summits near another summit less likely to make contact themselves, thus breaking up the contact area into smaller patches.

The importance of multiscale roughness has long been recognized (9), but the Contact Mechanics Challenge results signal that the time has come to standardize multiscale descriptions with elastic coupling, to develop next-generation models to account for behavior beyond the elastic limit, and to confront the next frontier in contact modeling-namely, describing rough surface contact during sliding. This last step would help fulfill da Vinci's dream of understanding what causes friction.

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ENVIRONMENTAL ECONOMICS

Air pollution's hidden impacts

Exposure can affect labor productivity and human capital

By Joshua Graff Zivin¹ and Matthew Neidell²

early every country in the world regulates air pollution. But how much pollution control is enough? Answering that question requires considerable information about the costs as well as the benefits of regulation. Historically, efforts to measure benefits have focused on averting major health insults, such as respiratory or cardiovascular events that result in hospitalizations or death, which typically only afflict the most vulnerable segments of the population. These health episodes are clearly consequential—e.g., the U.S. Clean Air Act Amendments of 1990 avert an estimated 160,000 deaths and 86,000 hospitalizations annually (1)—but may only represent the tip of the proverbial iceberg, compared to the number of cases of respiratory impairment and other health insults that affect many healthy people every day but do not require hospitalizations or even formal health care encounters. The ubiquity of these less lethal impacts, revealed by emerging economic research on labor productivity and human capital accumulation, suggests that even modest impacts at the individual level can add up to considerable, society-wide impacts across the globe.

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Our understanding of these less-severe health impacts is hampered by two factors. First, the number of potential channels through which pollution could affect human functioning is large, as pollution can alter the function of several organ systems and even genetic expression (2). Because these are difficult to assess, only a relatively small number have been well studied, leaving large gaps in our understanding of basic physiological relationships. Second, among channels that have been studied, it is often difficult to discern how the physiological and cognitive impacts that have been identified translate into meaningful effects outside of the controlled laboratory setting in which they were assessed. Does a 10% drop in forced expiratory volume, for example, influence the performance of daily life activities?

An emerging literature has begun to overcome these challenges with a new focus on the effects of acute exposure on measures of great economic importance, which, in turn, can easily be monetized for regulatory purposes.

LABOR MARKET PERFORMANCE

Although hospitalized individuals are unable to attend work, and others who are severely ill may also miss workdays, the impacts from less-severe pollution-induced illness on work hours are less well known. Research in Mexico City found that reductions in sulfur dioxide pollution led to sizable increases in work hours per week by individuals in the local

labor market (3). Of course, the degree of infirmity that necessitates a shortened workday is still nontrivial. It may require some type of health care encounter and at a minimum is obvious to the person experiencing it.

A handful of recent studies attempt to capture even subtler health impacts by focusing on the productivity of workers while they work. The premise behind these studies is that even minor impairments of respiratory and cardiovascular function can increase fatigue, decrease focus, and impair cognition (4), even in seemingly healthy populations, thus diminishing the ability to perform one's job. For example, agricultural workers in the Central Valley of California produce less as ozone increases, even though daily pay directly depends on how much fruit they harvest (5). These effects arise at ambient concentrations of ozone that are well below regulatory standards and at levels at which obvious health symptoms are not generally present in healthy populations.

Although agricultural employment is important in developing countries, it represents a small fraction of the labor force in more developed ones. Moreover, because agricultural work takes place outdoors, it is not a priori obvious that these results are germane to indoor work, which contributes most of the value in most economies. But a recent study and some preliminary findings suggest impacts of particulate matter pollution on indoor worker productivity in the manufacturing sector (6, 7).

The aforementioned studies focus on physically demanding work, and thus perhaps represent the most logical place for the impacts of minor respiratory and cardiovascular insults to manifest themselves. But findings from a study in China suggest that the information economy is not inoculated against these effects (8). Productivity of call center workers in two Chinese cities is negatively affected by fine particulate matter (particles with diameters less than 2.5 μm). These impacts are not limited to extreme pollution days, but emerge at pollution levels that regularly obtain in several major cities within the United States.

HUMAN CAPITAL ACCUMULATION

Human capital, a measure of the intangible resources an individual possesses, such as knowledge, skills, and judgment, plays a fundamental role in labor market outcomes and other aspects of life, including health, civic participation, and criminal activities. A fairly new body of literature focuses on latent impacts of in utero and early-childhood exposure to pollution on later-life outcomes. Although health insults from pollution may indirectly affect human capital accumulation through channels like school attendance and

other educational investments, this focus is motivated by the "fetal origins hypothesis" and literature that documents impacts of stimuli during these critical periods of human development (9).

Recent studies provide compelling evidence that early-life exposure to pollution can have lasting impacts on cognitive abilities later in life. Children born in Texas when airborne total suspended particulates (TSPs) were atypically low (due to decreases in industrial production during a brief economic recession) went on to have higher high school test scores (10). Children born in Santiago, Chile, with higher exposure to fetal pollution, such as carbon monoxide and particulate matter, went on to perform worse on high-stakes national exams that determine access to secondary schools (11). Another study makes the linkage between the labor productivity and human capital literatures more transparent, demonstrating that higher TSP levels in the year of birth lead to lower labor force participation and lower earnings in adulthood (12).

INCENTIVES AND DESIGN

Much of what is currently known about benefits from environmental protection in the United States is a result of measurement incentives embodied in Section 812 of the 1990 Clean Air Act amendments and other executive orders that require cost-benefit analyses. U.S. Environmental Protection Agency (EPA) procedures virtually ensure that those incentives remain focused on nationally representative evidence of impacts on high-visibility health endpoints, leaving the investigation of a wide range of other impacts to the idiosyncratic curiosities of the research community. These new forms of evidence could be incorporated into the formal structure of EPA cost-benefit analyses (13), while recognizing that forgone earnings due to diminished labor productivity or human capital attainment will not capture all the welfare losses experienced.

More can be done to transform opportunistic study of individual firms into systematic study of productivity impacts. Existing national surveillance systems can be augmented to collect data from industries in which productivity measures are standardized, such as local, state, and national employment data collected from the U.S. Bureau of Labor Statistics. Such data will facilitate calculations of benefits, play a critical role in assessing the generalizability of the results thus far uncovered, and provide a database through which new channels of influence can be explored. For industries in which productivity measures are not standardized. national data on worker hours and absences that could be matched to environmental data could prove useful in uncovering morbidity impacts and how they affect labor markets.

The ideal data collection effort would provide rich spatial and temporal resolution at the level of individual workers. Because such data would provide valuable intelligence on the state of the economy more generally, it would naturally fall in the United States under the jurisdiction of the Departments of Labor or Commerce. Absent resources for substantial new data collection efforts, some of these data could be collected through supplements to existing efforts by the Census Bureau, including the Annual Survey of Manufacturers and the every-5-year Economic Census for industries outside of manufacturing.

The impacts of pollution on labor markets and human capital suggest that pol-

"The impacts of pollution on labor markets and human capital suggest that policy implications extend beyond the EPA..."

icy implications extend beyond the EPA, transforming part of this problem into an occupational safety issue. As such, the Occupational Safety and Health Administration, both in the United States and European Union and in similar agencies in many other countries, may have a role to play in improving indoor air-quality monitoring in the workplace and fostering solutions to mitigate these impacts. If made public, these indoor air-quality measures could help overcome an important obstacle in current research and help deepen our understanding of the relationships between pollution, health, and worker productivity.

The long-lived impacts of pollution exposure during a brief window in early life also suggest a potential role for public health interventions that target pregnant mothers and underscore the need for more epigenetic research to uncover the mechanisms through which these impacts manifest themselves. Although the science is not completely settled. this relatively brief and well-identified period of vulnerability appears particularly ripe for small-scale interventions that can minimize exposure with large-scale returns. Health care providers and public health officials can play a more prominent role in disseminating relevant information and in facilitating access to technologies, such as home air filters, designed to eliminate harm.

Advances in information technology and measurement have allowed examination of a wide range of pollution impacts that were

not visible even a decade ago. Yet, in many respects, these studies just begin to scratch the surface. Much is still unknown about how far these impacts might reach. If pollution can affect reasoned judgment and decision-making, as some recent preliminary findings suggest (14), then perhaps every aspect of daily living may be altered by our contaminated environment. Whether these effects of acute exposure to pollution are compounded by chronic exposure is also of great importance and is largely unknown.

Deepening our scientific understanding of these relationships is constrained by the nature of the exercise, which tends to fall between the conventional silos of major research funding agencies. The EPA spends relatively little money on external research. The health-centric focus of the National Institute of Environmental Health Science (NIEHS) has generally viewed this more expansive view of health with a skeptical eye. The Dynamics of Coupled Natural and Human Systems Program at the National Science Foundation (NSF) remains focused on a much broader scale than the studies outlined above. Broadening the scope of what constitutes health at the NIEHS, a more humanscale focus on environmental impacts at the NSF, and a more robust extramural research program at the EPA could help advance scientific understanding of these relationships.

The societal harm generated by these more subtle and pervasive impacts should be reflected in the calculus that helps to determine regulatory standards. Whether they are large enough to justify further revisions to those standards is an important question worthy of more effort and attention.

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PHYSICS

The art of space-time

An ambitious exhibition explores the intersection of the cosmos and contemporary artistry

Gravity. Imaging the

Universe After Einstein

National Museum of the

21st Century Arts Rome, Italy.

Through 29 April 2018.

By Edwin Cartlidge

hen it comes to sparking the public's interest in science, no individual, living or dead, can rival Albert Einstein. His name and face, if not his ideas, are known the world over, and headline writers cite him eagerly to pull in readers. So perhaps it is no surprise that the Museo Nazionale delle Arti del XXI Secolo (MAXXI; National Museum of 21st Century Arts) in Rome has chosen the famous physicist as the figurehead of its latest exhibition, enti-

tled Gravity. Imaging the Universe After Einstein.

Einstein's theory of gravity, known as general relativity, marks a fundamental break in our conception of the universe. Whereas Newton envisaged gravity as a force of attraction between two objects in empty

space, relativity instead tells us that gravity is a distortion of space (and time) itself. The theory was published in 1915 and since then has been confirmed experimentally time and again, including the spectacular, if expected, discovery in 2015 of ripples in space-time known as gravitational waves.

The MAXXI exhibition, running until 29 April 2018, is designed to study "the meeting point of the current understanding of the cos-

in the words of MAXXI president Giovanna Melandri, who dreamt up the event, to show that art and science are "two lenses giving a single vision." Co-organizer Fernando Ferroni, president of Italy's National Institute of Nuclear Physics, echoes that sentiment. "Specialization in itself doesn't resolve anything," he says. "You need a big vision of society." Whether the exhibition gives visitors that

mos and contemporary art and thinking." Or,

vision is debatable. The centerpiece of the show is an installation by Argentine artist Tomás Saraceno entitled "Cosmic Concert" that cleverly portrays how we influence the

> space-time structure of the universe just as space-time affects us. It consists of a spider slowly weaving its web, which quivers ever so slightly in response to changing sounds and other vibrations in the environment while its tiny movements are themselves picked up by sensors

that in turn modify the output from an array of loudspeakers overhead.

Elsewhere, however, art seems thin on the ground. In one corner of the exhibition gallery sits a model of the New Jersey radio antenna that in 1965 provided the first evidence of the cosmic microwave background, very faint but ubiquitous radiation emitted just after the universe exploded into life during the Big Bang. Accompanied by a mildly hypnotic soundtrack and projection showing what appear to be bats flying through a forest, the strangely shaped device, alternately An interactive video installation allows visitors to deform space-time and generate gravitational waves.

illuminated by the video and in shade, is intriguing but inert. More entertaining is footage showing an interlinked series of things (e.g., bottles, tires, candles, and balloons) rolling down planks, spilling, burning, and exploding-an "endless chain reaction of apparently insignificant events" that is thoughtprovoking but slightly at odds with the other items on display.

Much of the exhibition consists of scientific instruments illuminated within the otherwise almost total darkness of the gallery. These artifacts include a mirror from the Virgo detector near Pisa, which, along with the Laser Interferometer Gravitational-Wave Observatory (LIGO) facility in the United States, uses laser beams as extremely precise rulers to measure the miniscule stretching and compression of space caused by passing gravitational waves. Also on display is a model of the Laser Relativity Satellite (LARES), a 36-centimeter-diameter tungsten sphere launched by the Italian Space Agency (ASI) to measure how a rotating body distorts space-time. There is even one of Galileo's telescopes, as well as a 17th-century armillary sphere showing the orbits of planets and moons in the solar system.

These objects have considerable scholarly and historical value, and their geometry and craftsmanship confer them with undoubted beauty. But they very much lie in the scientific, rather than the artistic, realm, as do the various explanations of scientific concepts dotted around the gallery, be they in the form of written text or in animations and interactive exhibits.

Some of those explanations will make more sense to the layman than others. One video graphic, illustrating how someone on a moving train measures space and time differently from someone standing still, is a brave stab at explaining the counterintuitive effects of special relativity in a simple way. But even this is likely to be beyond the conceptual ability of most museumgoers.

Surely the aim of an exhibition like this is to pique people's curiosity and give them a very broad sense of the mind-bending concepts that underlie modern physics. The art, if well done, could whet their appetite and, as ASI's Roberto Battiston put it, "expand their horizons." But too much detail is likely to turn them off and reinforce their impression that physics is only for the eggheads, not for them. Pulling off a true synthesis of art and science is always hard, and unfortunately, with Gravity, MAXXI has fallen some way short.

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10.1126/science.aar6576

Social science, today

A breezy, personal guide provides a road map to solid computational social science research

By David Lazer

ubatomic particle physics has CERN. Astronomy has the Hubble telescope. Social science has the Internet, smartphones, email, social media, satellites, and a myriad of other ways to follow human behavior. The gods of the information age have produced a whole panoply of technologies for social research along the journey to other destinations.

Generally, social scientists have been poorly equipped to deal with the 21st-century deluge of large-scale complex data. Computer scientists, well equipped to handle the data, are often ignorant of social theory and of foundational research methods in the social sciences. What is needed is an articulation of core principles of designing research that are accessible to multiple disciplines.

Into this breach steps Matthew Salganik. Salganik is one of the first natural-born computational social scientists, a sociologist whose doctoral work was one of the early landmark projects in the field (1). Bit by Bit is 90% textbook, 10% biography, putting into personal context issues that Salganik was among the first to wrestle with.

The volume fits solidly into a genre of textbooks in the social sciences, inaugurated in

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1963 by Donald Campbell and Julian Stanley (2), that might be termed "practical epistemology." That is, they address such questions as these: How do we create knowledge in the social sciences? How do we measure things? What's the basis for statements like "20% of the population is X", or "X causes Y"?

In a sense, this type of textbook is about the architecture of research, the structure that will be necessary to support potential assertions. However, the range of social science research activities has changed

dramatically, with profound implications for potential design.

Much as a steel frame enables the construction of buildings that reach toward the heavens and transform city skylines, the pervasive instrumentation of human behavior should likewise transform social science research. Bit by Bit is the first-and quite worthy-successor to the Campbell volume, aimed at informing students how to design those blueprints in the emerging field of computational social science.

Other than the introduction and conclusion, there are five core chapters to Bit by Bit. The first, "Observing behavior," is foodology to big data: Core concerns about representativeness and measurement are amplified when recycling big data collected for other purposes.

"Running experiments" discusses the scientific potential to run heretofore inconceivably large-scale experiments. The Internet, in particular, argues Salganik, enables the facilitation of large group experiments as well as the evaluation of heterogeneity of treatment effects. "Creating mass collaboration" discusses the harnessing of the small efforts of many people for large-scale scientific applications.

The final chapter, "Ethics," is a thoughtful exposition on the core principles around ethical research generally, with a particular focus on the challenges that large-scale data collection poses. Privacy and security concerns, for example, are magnified with scale and consentless third-party data collection.

> The text is clearly writteneven breezy, in parts. It puts the reader in the shoes of the researcher: What decisions were made, why, and were those the best choices? It is suitable for an advanced undergraduate or graduate class in methodology, with a rigorous, mathematical appendix and a range of useful problems at the conclusion of each chapter.

> This book is not the place to learn about cutting-edge computational techniques. However, if

vou want to reflect on the potential value of. say, deep learning to understanding human behavioral data, there are relevant lessons. Despite the rapid evolution of the domain, this book will likely have staying power.

It is telling that my only complaint is that I would have liked to see more topics covered. How, for example, do we translate more quasi-experimental approaches to the big data world? How do we rethink the power of panel data when there may be thousands or millions of observations per individual? How do we manage the complex workflow of a computational social science project? How do we deal with the issue of replication with data that often cannot be shared?

It may be, however, that the field more generally must advance before these chapters can be written. In the interim, Bit by Bit will be required reading for my students.



Bit by Bit

Social Research in

the Digital Age

Matthew J. Salganik

Princeton University

Press, 2017. 445 pp.

- 1. M. J. Salganik, P. S. Dodds, D. J. Watts, Science 311, 854 (2006)
- D. Campbell, J. Stanley, Experimental and Quasi-Experimental Designs for Research (Houghton Mifflin, Boston, 1963)

cused on the massive, passive data collection that occurs in everyday life, identifying key opportunities and challenges in using big data for research. "Asking questions" adapts lessons learned from survey meth-

A deluge of digital data is transforming the way we collect and interpret social research.

RESEARCH

Vivax malaria uses transferrin receptor to gain entry

Gruszczyk et al., p. 48



IN SCIENCE JOURNALS

Edited by Stella Hurtley



GAS GIANT PLANETS

Cassini enters Saturn's ionosphere

he upper reaches of most planetary atmospheres contain a layer that is ionized by incoming solar radiation—the ionosphere. As it went through its final orbits around Saturn, the Cassini spacecraft dipped close enough to the planet to pass directly through the ionosphere. Wahlund et al. examined the plasma data collected in situ and found that Saturn's ionosphere is highly variable and interacts with the planet's inner ring. They also observed decreases in ionization within regions shaded from the Sun by the rings. —KTS

Science, this issue p. 66

MATERIALS SCIENCE

Liquids show their strength

Dielectric elastomer actuators are electrically powered muscle mimetics that offer high actuation strain and high efficiency but are limited by failure caused by high electric fields and aging. Acome et al. used a liquid dielectric, rather than an elastomeric polymer, to

solve a problem of catastrophic failure in dielectric elastomer actuators. The dielectric's liquid nature allowed it to self-healsomething that would not be possible with a solid dielectric. The approach allowed the authors to exploit electrostatic and hydraulic forces to achieve muscle-like contractions in a powerful but delicate gripper.

Science: this issue p. 61

TOPOLOGICAL MATTER

Heating up the quantum spin Hall effect

Taking practical advantage of the topologically protected conducting edge states of topological insulators (TIs) has proven difficult. Semiconductor systems that have been identified as twodimensional TIs must be cooled down to near liquid helium temperatures to bring out their

topological character. Wu et al. fabricated a heterostructure consisting of a monolayer of WTe, placed between two layers of hexagonal boron nitride and found that its topological properties persisted up to a relatively high temperature of 100 K. Engineering this so-called quantum spin Hall effect in a van der Waals heterostructure makes it possible to apply many established experimental tools and functionalities. -JS

Science, this issue p. 76

CANCER IMMUNOTHERAPY

Good bacteria help fight cancer

Resident gut bacteria can affect patient responses to cancer immunotherapy (see the Perspective by Jobin). Routy et al. show that antibiotic consumption is associated with poor response to immunotherapeutic PD-1 blockade. They profiled samples from patients with lung and kidney cancers and found that nonresponding patients had low levels of the bacterium Akkermansia muciniphila. Oral supplementation of the bacteria to antibiotic-treated mice restored the response to immunotherapy. Matson et al. and Gopalakrishnan et al. studied melanoma patients receiving PD-1 blockade and found a greater abundance of "good" bacteria in the guts of responding patients. Nonresponders had an imbalance in gut flora composition, which correlated with impaired immune cell activity. Thus, maintaining healthy gut flora could help patients combat cancer. -PNK

> Science, this issue p. 91, p. 104, p. 97; see also p. 32

MOLECULAR BIOLOGY

Mitosis-specific role of ATR

The ATR (ataxia telangiectasia mutated and Rad3-related) kinase plays important roles in the S phase and during the DNA damage response to safeguard genome integrity. Kabeche et al. identified a distinct ATR activation pathway in mitosis that is also critical for suppressing genome instability (see the Perspective by Saldivar and Cimprich). ATR is recruited by Aurora A and activated by R loops at centromeres of mitotic chromosomes; this leads to Aurora B activation, which is necessary for accurate chromosome segregation. This mitotic, R loop-driven ATR signaling pathway could potentially be exploited in the search for cancer therapeutics. —SYM

> Science, this issue p. 108; see also p. 30

CORAL REEFS

Not enough time for recovery

Coral bleaching occurs when stressful conditions result in the expulsion of the algal partner from the coral. Before anthropogenic climate warming, such events were relatively rare, allowing for recovery of the reef between events. Hughes et al. looked at 100 reefs globally and found that the average interval between bleaching events is now less than half what it was before. Such narrow recovery windows do not allow for full recovery. Furthermore, warming events such as El Niño are warmer than previously, as are general ocean conditions. Such changes are



In a warming world, corals struggle to recover between bleaching events.

likely to make it more and more difficult for reefs to recover between stressful events. -SNV Science, this issue p. 80

SOCIAL SCIENCES

Global reciprocity drives cooperation

Cooperation among nations promotes international trade, law, peace, and environmental protection. But how does cooperation emerge and persist among independent, selfinterested, and often competing nations? Frank et al. applied powerful causal inference techniques to a detailed global data set of country interactions from 1995 to 2015 to map international cooperation, influence, and reciprocity. In agreement with predictions from evolutionary game theory, reciprocity among nations, including powerful countries, is pervasive and leads to stable cooperation, even in the face of minor transgressions. -AC

> Sci. Adv. 10.1126/ sciadv.aao5348 (2018).

TINNITUS

The sound of silence

Tinnitus reduces the quality of life for millions of sufferers worldwide. Using a guinea pig model of tinnitus induced by noise trauma, Marks et al. delivered precisely timed bimodal auditory-somatosensory stimulation to induce long-term depression (LTD) in the cochlear nucleus. Twenty minutes of treatment per day reduced physiological and behavioral evidence of tinnitus in the animals.

> The same bimodal protocol reduced tinnitus loudness in human subjects in a doubleblind, sham-controlled, crossover clinical study. Unimodal stimulation did not reduce tinnitus in the animals or the humans. This approach thus holds promise for suppressing chronic tinnitus in patients. —OMS

Sci. Transl. Med. 10, eaal3175 (2018).



SIGNAL TRANSDUCTION

Unconventional thyroid hormone signals

Thyroid hormone canonically signals through thyroid hormone receptors to enhance transcription of target genes. There is also evidence that thyroid hormone can activate nontranscriptional signaling mechanisms. To sort out the relative importance of canonical and noncanonical signaling; Hones et al. generated mice in which thyroid hormone receptors were altered to prevent DNA binding and transcriptional effects (but noncanonical signaling remained) and compared thyroid hormone action in these animals with that in wild-type mice or mice lacking thyroid hormone receptors entirely. They found that in vivo, several physiological actions of thyroid hormone—including regulation of body temperature, glucose and triglyceride concentrations in the blood, and heart rate—all appear to be mediated by noncanonical or nontranscriptional mechanisms. -LBR

Proc. Natl. Acad. Sci. U.S.A. 10.1073/ pnas.1706801115 (2017).

BIOCATALYSIS

Guiding an enzyme all around a ring

Most compounds of interest for pharmaceuticals, agrochemicals, and cosmetics have many C-H bonds, interspersed with a few carbon bonds to heavier elements that give them their distinct properties. Chemists therefore prize methods that let them selectively modify a variety of C-H bonds. Gilbert et al. report a versatile strategy that relies on a tethered amine to steer an engineered cytochrome P450 enzyme around 11- or 12-membered rings, transforming specific C-H bonds into C-O bonds. Subtle structural variation of the tether through click chemistry tunes the site selectivity. - JSY

ACS Cent. Sci. 10.1021/ acscentsci.7b00450 (2017).

MINOR PLANETS

An object from beyond the solar system

Gravitational interactions occasionally eject small bodies from the solar system, and this process is thought to have been



PARASITE GENOMICS

Single-cell sequencing of malarial genomes

nfection with the parasite Plasmodium falciparum causes malaria. Individuals may be infected with multiple strains of P. falciparum, some of which may be drug-resistant. Understanding the complexity of these infections may provide information about the diversity of the parasite population and aid in drug-targeting strategies. Overcoming the difficulties caused by AT bias in the Plasmodium genome, Trevino et al. optimized single-cell sequencing for late-stage P. falciparum parasites within infected individuals. From this analysis, they documented at least seven distinct parasitic haplotypes, traced meiotic events and hence relatedness among parasitic lineages, and found distinct singlenucleotide variants that could be used for bulk analysis. -LMZ

Genome Biol. Evol. 10.1093/gbe/evx256 (2017).

Single-cell sequencing of Plasmodium, here released from lysed red blood cells, allows for tracking of variation in the host.

particularly common while the system was first forming. The same thing should have occurred around other stars, casting comets and asteroids into interstellar space, but no such interstellar objects have been identified. Meech et al. discovered a small object, now formally designated 11/2017 U1 ('Oumuamua), whose trajectory indicates that it arrived from interstellar space. Changes in its brightness demonstrate that 'Oumuamua is rotating and is several times longer than it is wide. 'Oumuamua is quickly passing through the solar system and soon will resume its interstellar journey. -KTS Nature 10.1038/nature 25020 (2017).

MICROBIOTA

Eats leaves and grooms

The gut microbiota influences many aspects of mammalian development and physiology. Yet we have a poor understanding of how the gut microbiota is acquired and assembled. We know that social networks are important for the transmission of pathogens, but are they also implicated in transmission

of symbionts? Perofsky et al. investigated the microbiota of a social primate, a lemur species called Verreaux's sifaka, living in the wild in Madagascar. These lemurs eat leaves and have a distinctive microbiota capable of digesting and detoxifying plant matter. The authors found that sex, dominance, and age influenced gut microbial

composition, but social group membership explained 58% of the variation. Grooming and scent-marking are essential for lemur social cohesion, and commensal microbes are transferred between individuals through this type of intimate contact, rather than being regulated by genetics or diet. -CA

Proc. R. Soc. B 284, 20172274 (2017)



Social interaction among Verreaux's sifaka is responsible for transmission of gut microbes that are essential for digestion of their leaf-based diet.

BIOLOGY

Cytoplasmic transfer to tumor cells

Macrophages are innate immune cells that, when recruited to tumors, can promote tumor progression. Macrophage activity and phenotype can be influenced by molecules secreted by tumor cells. To monitor macrophage behavior, Roh-Johnson et al. used time-lapse imaging of a zebrafish model of melanoma. Unexpectedly, macrophages not only bound to but also transferred their cytoplasm into melanoma cells, which promoted metastasis. Macrophage cytoplasmic transfer to melanoma cells also correlated with metastasis in mice. This unusual mechanism of cell-cell signaling raises questions about how such intercellular communication is regulated and what molecules are transferred to melanoma cells to promote their metastasis. -GKA

Dev. Cell 43, 549 (2017).

TRANSLATION

Ribosomes ignore the stop sign

Aminoglycoside antibiotics bind to bacterial ribosomes and inhibit protein synthesis. Eukaryotic ribosomes, in contrast, are not strongly inhibited by these molecules but show errors such as inaccurate translation and read-through of stop codons. Prokhorova et al. determined structures of the eukaryotic ribosome in complex with aminoglycoside antibiotics and investigated how these molecules alter the conformation of the ribosome. They found distinct binding sites for different classes of aminoglycosides and multiple possible binding sites on each ribosome. The conformational changes induced by these molecules suggest multiple mechanisms by which the antibiotics interfere with eukaryotic translation. -MAF

Proc. Natl. Acad. Sci. U.S.A. 10.1073/ pnas.1715501114 (2017).

REVIEW SUMMARY

OCEANS

Declining oxygen in the global ocean and coastal waters

Denise Breitburg,* Lisa A. Levin, Andreas Oschlies, Marilaure Grégoire, Francisco P. Chavez, Daniel J. Conley, Véronique Garçon, Denis Gilbert, Dimitri Gutiérrez, Kirsten Isensee, Gil S. Jacinto, Karin E. Limburg, Ivonne Montes, S. W. A. Naqvi, Grant C. Pitcher, Nancy N. Rabalais, Michael R. Roman, Kenneth A. Rose, Brad A. Seibel, Maciej Telszewski, Moriaki Yasuhara, Jing Zhang

BACKGROUND: Oxygen concentrations in both the open ocean and coastal waters have been declining since at least the middle of the 20th century. This oxygen loss, or deoxygenation, is one of the most important changes occurring in an ocean increasingly modified by human activities that have raised temperatures, CO2 levels, and nutrient inputs and have altered the abundances and distributions of marine species. Oxygen is fundamental to biological and biogeochemical processes in the ocean. Its decline can cause major changes in ocean productivity, biodiversity, and biogeochemical cycles. Analyses of direct measurements at sites around the world indicate that oxygen-minimum zones in the open ocean have expanded by several million square kilometers and that hundreds of coastal sites now have oxygen concentrations low enough to limit the distribution and abundance of animal populations and alter the cycling of important nutrients.

ADVANCES: In the open ocean, global warming, which is primarily caused by increased primary cause of ongoing deoxygenation. Numerical models project further oxygen declines during the 21st century, even with ambitious emission reductions. Rising global temperatures decrease oxygen solubility in water, increase the rate of oxygen consumption via respiration, and are predicted to reduce the introduction of oxygen from the atmosphere and surface waters into the ocean interior by increasing stratification and weakening ocean overturning circulation.

In estuaries and other coastal systems strongly influenced by their watershed, oxygen declines have been caused by increased loadings of nutrients (nitrogen and phosphorus) and organic matter, primarily from agriculture; sewage; and the combustion of fossil fuels. In many regions, further increases in nitrogen discharges to coastal waters are projected as human populations and agricultural production rise. Climate change exacerbates oxygen decline in coastal systems through similar mechanisms as those in the open ocean, as well as by increasing nutrient

greenhouse gas emissions, is considered the

Hypoxic areas

Low and declining oxygen levels in the open ocean and coastal waters affect processes ranging from biogeochemistry to food security. The global map indicates coastal sites where anthropogenic nutrients have exacerbated or caused O_2 declines to <2 mg liter⁻¹ (<63 μmol liter⁻¹) (red dots), as well as ocean oxygen-minimum zones at 300 m of depth (blue shaded regions). [Map created from data provided by R. Diaz, updated by members of the GO₂NE network, and downloaded from the World Ocean Atlas 2009].

delivery from watersheds that will experience increased precipitation.

Expansion of low-oxygen zones can increase production of N2O, a potent greenhouse gas; reduce eukaryote biodiversity; alter the structure of food webs; and negatively affect food security and livelihoods. Both acidification and increasing temperature are mechanistically linked with the process of deoxygenation and combine with low-oxygen conditions to affect biogeo-

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chemical, physiological, and ecological processes. However, an important paradox to consider in predicting large-scale effects of future deoxygenation is that high levels of productivity in

nutrient-enriched coastal systems and upwelling areas associated with oxygen-minimum zones also support some of the world's most prolific fisheries.

OUTLOOK: Major advances have been made

toward understanding patterns, drivers, and consequences of ocean deoxygenation, but there is a need to improve predictions at large spatial and temporal scales important to ecosystem services provided by the ocean. Improved numerical models of oceanographic processes that control oxygen depletion and the large-scale influence of altered biogeochemical cycles are needed to better predict the magnitude and spatial patterns of deoxygenation in the open ocean, as well as feedbacks to climate. Developing and verifying the next generation of these models will require increased in situ observations and improved mechanistic understanding on a variety of scales. Models useful for managing nutrient loads can simulate oxygen loss in coastal waters with some skill, but their ability to project future oxygen loss is often hampered by insufficient data and climate model projections on drivers at appropriate temporal and spatial scales. Predicting deoxygenation-induced changes in ecosystem services and human welfare requires scaling effects that are measured on individual organisms to populations, food webs, and fisheries stocks; considering combined effects of deoxygenation and other ocean stressors; and placing an increased research emphasis on developing nations. Reducing the impacts of other stressors may provide some protection to species negatively affected by lowoxygen conditions. Ultimately, though, limiting deoxygenation and its negative effects will necessitate a substantial global decrease in greenhouse gas emissions, as well as reductions in nutrient discharges to coastal waters.

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RESEARCH ARTICLE SUMMARY

CELL BIOLOGY

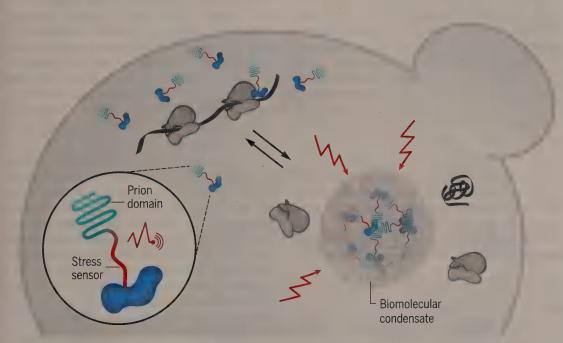
Phase separation of a yeast prion protein promotes cellular fitness

Titus M. Franzmann, Marcus Jahnel, Andrei Pozniakovsky, Julia Mahamid, Alex S. Holehouse, Elisabeth Nüske, Doris Richter, Wolfgang Baumeister, Stephan W. Grill, Rohit V. Pappu, Anthony A. Hyman,* Simon Alberti*

INTRODUCTION: The formation of dynamic, membraneless compartments using intracellular phase transitions such as phase separation and gelation provides an efficient way for cells to respond to environmental changes. Recent work has identified a special class of intrinsically disordered domains enriched for polar amino acids such as glycine, glutamine, serine, or tyrosine as potential drivers of phase separation in cells. However, more traditional work has highlighted the ability of these domains to drive the formation of fibrillar aggregates. Such domains are also known as prion domains. They have first been identified in budding yeast proteins that form amyloidlike aggregates. Because these aggregates are heritable and change the activity of the priondomain-containing protein, they are thought to be a common mechanism for phenotypic inheritance in fungi and other organisms. How-

ever, the aggregation of prion domains has also been associated with neurodegenerative diseases in mammals. Therefore, the relationship between the role of these domains as drivers of phase separation and their ability to form prionlike aggregates is unknown.

RATIONALE: The budding yeast translation termination factor Sup35 is an archetypal priondomain-containing protein. Sup35 forms irreversible heritable aggregates, and these aggregates have been proposed to be either a disease or an adaptation that generates heritable phenotypic variation in populations of budding yeast. Despite having been described almost 25 years ago, the physiological functions of the Sup35 prion domain and other prion-like domains remain unclear. Uncovering these functions is a prerequisite for understanding the evolutionary pressures shaping prion-like se-



The Sup35 prion domain regulates phase separation of the translation termination factor Sup35 during cellular stress. The translation termination factor Sup35 (depicted in the magnifying glass) consists of a disordered prion domain (cyan) a disordered stress sensor domain (red) and a folded catalytic domain (blue). During growth, Sup35 catalyzes translation termination. During cell stress, the prion domain and the sensor domain act together to promote phase separation into protective and reversible biomolecular condensates.

quences and how their physiological and pathological transitions affect cellular fitness.

RESULTS: Here, we show that the prion domain of Sup35 drives the reversible phase separation of the translation termination factor into biomolecular condensates. These condensates are distinct and different from fibrillar amyloid-like prion particles. Combining genetic analysis in cells with in vitro reconstitution

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protein biochemistry and quantitative biophysical methods, we demonstrate that Sup35 condensates form by pH-induced liquidlike phase separation as a response to sudden stress.

The condensates are liquid-like initially but subsequently solidify to form protective protein gels. Cryo-electron tomography demonstrates that these gel-like condensates consist of crosslinked Sup35 molecules forming a porous meshwork. A cluster of negatively charged amino acids functions as a pH sensor and regulates condensate formation. The ability to form biomolecular condensates is shared among distantly related budding yeast and fission yeast. This suggests that condensate formation is a conserved and ancestral function of the prion domain of Sup35. In agreement with an important physiological function of the prion domain, the catalytic guanosine triphosphatase (GTPase) domain of the translation termination factor Sup35 readily forms irreversible aggregates in the absence of the prion domain. Consequently, cells lacking the prion domain exhibit impaired translational activity and a growth defect when recovering from stress. These data demonstrate that the prion domain rescues the essential GTPase domain of Sup35 from irreversible aggregation, thus ensuring that the translation termination factor remains functional during harsh environmental conditions.

CONCLUSION: The prion domain of Sup35 is a highly regulated molecular device that has the ability to sense and respond to physiochemical changes within cells. The N-terminal prion domain provides the interactions that drive liquid phase separation. Phase separation is regulated by the adjacent stress sensor. The synergy of these two modules enables the essential translation termination factor to rapidly form protective condensates during stress. This suggests that prion domains are protein-specific stress sensors and modifiers of protein phase transitions that allow cells to respond to specific environmental conditions.

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RESEARCH ARTICLES

MALARIA

Transferrin receptor 1 is a reticulocyte-specific receptor for Plasmodium vivax

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Plasmodium vivax shows a strict host tropism for reticulocytes. We identified transferrin receptor 1 (TfR1) as the receptor for P. vivax reticulocyte-binding protein 2b (PvRBP2b). We determined the structure of the N-terminal domain of PvRBP2b involved in red blood cell binding, elucidating the molecular basis for TfR1 recognition. We validated TfR1 as the biological target of PvRBP2b engagement by means of TfR1 expression knockdown analysis. TfR1 mutant cells deficient in PvRBP2b binding were refractory to invasion of P. vivax but not to invasion of P. falciparum. Using Brazilian and Thai clinical isolates, we show that PvRBP2b monoclonal antibodies that inhibit reticulocyte binding also block P. vivax entry into reticulocytes. These data show that TfR1-PvRBP2b invasion pathway is critical for the recognition of reticulocytes during P. vivax invasion.

f the hundreds of Plasmodium species, only P. falciparum, P. vivax, P. ovale curtisi, P. ovale wallikeri, P. malariae, and P. knowlesi are known to infect humans. Within the human host, malaria parasites invade liver and red blood cells for replication and transmission. Blood stage infection is the major cause of all clinical symptoms in malaria, and therefore the therapeutic prevention of parasite entry into red blood cells could alleviate malarial disease. Entry into red blood cells depends on the interactions between parasite invasion ligands and their cognate red blood cell receptors, of which only a handful have been identified (1-7). These ligand-receptor interactions initiate a cascade of molecular events that progress from initial attachment, recognition, commitment, and last, penetration of the parasite into red blood cells (8, 9).

P. vivax is the most widely distributed human malaria parasite. This parasite has a strict preference for invasion into reticulocytes, which are very young red blood cells that are formed in the bone marrow after enucleation and released into the circulation. The reticulocyte-specific receptor involved in P. vivax entry has not been identified (10). Most studies have focused on the interaction between the P. vivax Duffy binding protein (PvDBP) and the red blood cell Duffy antigen receptor for chemokines (DARC) because individuals from western and central Africa lacking DARC are resistant to P. vivax invasion (11). However, recent reports have highlighted the presence of P. vivax in apparently DARCnegative individuals, suggesting that P. vivax may enter reticulocytes by binding to other receptors (12-14). Furthermore, DARC is present on both normocytes and reticulocytes, and therefore this ligand-receptor interaction cannot govern selective entry into reticulocytes (15). To identify other parasite proteins involved in reticulocyte recognition, we focused on the P. vivax

reticulocyte-binding protein family (PvRBP). This protein family comprises 11 members, of which several have been shown to bind reticulocytes; however, their cognate receptors have not been identified (16-19).

PvRBP2b binds transferrin receptor 1 to mediate recognition of reticulocytes

P. vivax preferentially invades reticulocytes that express high levels of transferrin receptor 1 (TfR1 or CD71) (20). TfR1 is an essential housekeeping protein involved in cellular transport of iron into cells through binding of iron-loaded transferrin (Tf) (21). On circulating red blood cells, TfR1 is expressed only on reticulocytes and is progressively lost from their membranes as they mature into erythrocytes (22, 23). TfR1 is a type II transmembrane glycoprotein that forms a dimer, and its ectodomain consists of three subdomains: a "protease-like domain" resembling the structure of zinc metalloproteinases, an "apical domain," and a "helical domain" responsible for dimerization (24). TfR1 is also a cellular receptor for New World hemorrhagic fever arenaviruses, including Machupo (MACV), Junin, Guanarito, and Sabiá viruses (25, 26). Residues 208 to 212 of the TfR1 apical domain provide a critical recognition site for these viruses (25, 26).

PvRBP2b is expressed in late-stage P. vivax parasites, and recombinant PvRBP2b (residues 161 to 1454; PvRBP2b₁₆₁₋₁₄₅₄) binds preferentially to reticulocytes that express TfR1 (19, 27). We observed that binding by recombinant PvRBP2b was abolished when reticulocytes were treated with trypsin and chymotrypsin (fig. S1, A and B). We confirmed that the combination of these proteases cleaves TfR1 and complement receptor 1 (CR1) from the surface of reticulocytes, with other known malaria receptors-including glycophorin A, basigin, and DARC-being susceptible to different sets of protease treatment (fig. S1, A and B). The profile of PvRBP2b binding is strikingly similar to the TfR1 surface expression on reticulocytes (Fig. 1A, bottom), and we show that the level of PvRBP2b binding is directly correlated with the levels of TfR1 on the surface of reticulocytes (fig. S1, C and D).

To determine whether PvRBP2b₁₆₁₋₁₄₅₄ binds to the population of reticulocytes that express TfR1 on their surfaces, we tested a panel of commercially available anti-TfR1 monoclonal antibodies (mAbs) for their ability to block recombinant PvRBP2b binding. Indeed, anti-TfR1 mAbs 23D10, LO1.1, LT71, M-A712, MEM-189, and OKT9 inhibited PvRBP2b binding to reticulocytes by 78, 76, 33, 75, 92, and 90%, respectively (Fig. 1A). M-A712 also prevents MACV pseudovirus entry (25, 28). Anti-TfR1 mAbs 2B6, 13E4, and MEM-75 did not inhibit PvRBP2b binding; although their epitopes have not been mapped, we propose that these three antibodies may bind to a site on TfR1 that is not involved in the PvRBP2b interaction (Fig. 1A). To determine whether this inhibition was specific to PvRBP2b₁₆₁₋₁₄₅₄ binding, we analyzed the binding of P. falciparum reticulocyte binding protein-like homolog 4 (PfRh4) to its cognate receptor CR1 (4). Whereas addition of the first three

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complement control protein modules of CR1 (CCP 1-3) inhibited PfRh4₂₈₋₇₆₆ binding as expected (29), addition of anti-TfR1 mAb OKT9 did not significantly reduce PfRh4 binding (Fig. 1B). Because anti-TfR1 did not affect PfRh4 binding, these results show that TfR1 is a specific reticulocyte receptor for PvRBP2b.

To evaluate whether PvRBP2b₁₆₁₋₁₄₅₄ interacts directly with TfR1, we performed immunoprecipitation experiments using purified recombinant TfR1, Tf, and PvRBP2b₁₆₁₋₁₄₅₄ proteins (Fig. 1C)

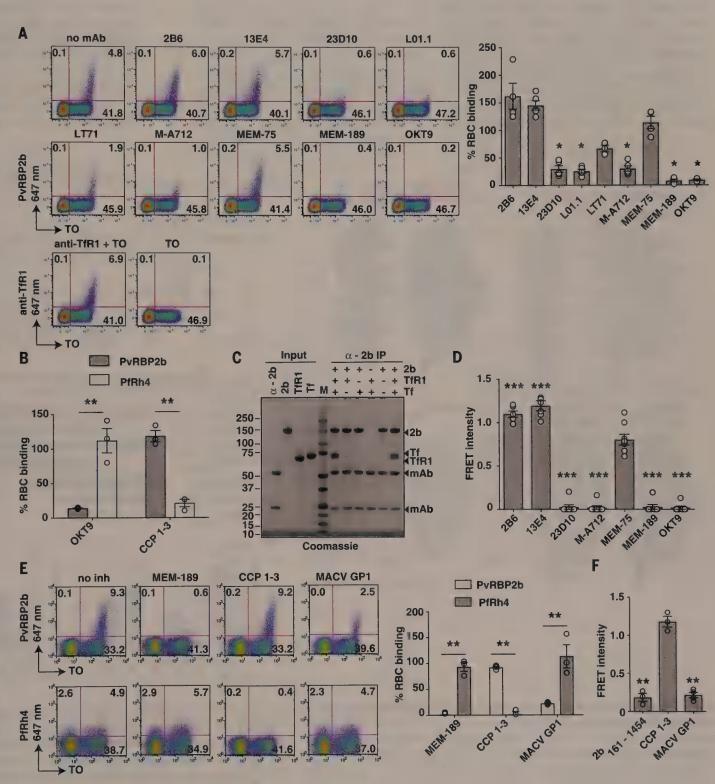
(30). Using an anti-PvRBP2b mAb, we immunoprecipitated PvRBP2b in complex with TfR1 and Tf. PvRBP2b and TfR1 also formed a binary complex in the absence of Tf, demonstrating that PvRBP2b binds directly to TfR1 (Fig. 1C). The interaction between PvRBP2b and TfR1 is specific; immunoprecipitation of PvRBP1a, PvRBP1b, or PvRBP2a did not show evidence of complex formation with TfR1 (fig. S2A).

We developed a fluorescence resonance energy transfer (FRET)-based assay to monitor PvRBP2b-

TfR1 complex formation in which TfR1 labeled with DyLight-594 could be shown to interact with PvRBP2b₁₆₁₋₁₄₅₄ labeled with DyLight-488 (fig. S2B). The addition of 10-fold molar excess of unlabeled PvRBP2b₁₆₁₋₁₄₅₄ and TfR1 competed out the labeled proteins and reduced the signal of the PvRBP2b-TfR1 FRET pair. By contrast, proteins that were unable to bind TfR1, such as PfRh4, had no effect on the FRET signal. Using this assay, we observed that anti-TfR1 mAbs 23D10, M-A712, MEM-189, and OKT9 that

Fig. 1. PvRBP2b binds TfR1 on the reticulocyte surface.

(A) PvRBP2b₁₆₁₋₁₄₅₄ binding in the presence of anti-TfR1 mAbs analyzed by means of flow cytometry. (Left) Dot plots of PvRBP2b₁₆₁₋₁₄₅₄ binding (y axis) to reticulocytes stained with thiazole orange (TO; x axis). (Right) Normalized binding results in which PvRBP2b₁₆₁₋₁₄₅₄ binding in the absence of mAbs was arbitrarily assigned to be 100%. (B) PvRBP2b₁₆₁₋₁₄₅₄ and PfRh4₂₈₋₇₆₆ binding were evaluated by means of flow cytometry with the addition of anti-TfR1 mAb OKT9 or CCP 1-3 PvRBP2b₁₆₁₋₁₄₅₄ and PfRh4 binding in buffer were arbitrarily assigned to be 100%. (C) Eluates of individual or mixtures of proteins immunoprecipitated with anti-PvRBP2b mAb analyzed by means of SDS-PAGE. Plus and minus signs indicate protein present and absent, respectively. M, molecular weight marker. (D) Anti-TfR1 mAbs inhibit PvRBP2b-TfR1 complex formation in the FRET-based assay. The FRET signal was relative to "no mAb"



PfRh4 $_{28-766}$ in the presence of anti-TfR1 mAb MEM-189, CCP 1–3, and MACV GP1. (Left) Dot plots showing PvRBP2b $_{161-1454}$ (top) and PfRh4 $_{28-766}$ binding (bottom). (Right) Normalized binding results in which PvRBP2b $_{161-1454}$ and PfRh4 $_{28-766}$ binding in the presence of buffer was arbitrarily assigned to be 100%. (**F**) MACV GP1 inhibits PvRBP2b $_{161-1454}$ –TfR1 complex

formation monitored by means of FRET assay. For (A), (B), (D), (E), and (F), mean \pm SEM, $n \geq 3$ biological replicates; open circles represent biological replicates. Mann-Whitney test was used for (A) and (D), where MEM-75 was considered noninhibitory, and t tests were used for (B), (E), and (F). $*P \leq 0.05$, $**P \leq 0.001$.

control. (**E**) Binding of PvRBP2b_{161–1454} and

inhibited PvRBP2b₁₆₁₋₁₄₅₄ reticulocyte binding also blocked PvRBP2b-TfR1 complex formation (Fig. 1D).

MACV GP1 and PvRBP2b bind to the apical domain of TfR1

The arenavirus envelope glycoprotein is the only protein on the virion surface and, during maturation, is processed into three subunits: the stable signal peptide, GP1, and GP2. The GP1 subunit interacts with cellular receptors, and the structure of a MACV GP1-TfR1 complex shows that MACV GP1 binds to the apical domain of TfR1 (31, 32). To determine whether PvRBP2b interacts with a similar surface on TfR1, we examined whether soluble MACV GP1 competes with PvRBP2b₁₆₁₋₁₄₅₄ for binding to TfR1 on reticulocytes (Fig. 1E). Indeed, the addition of MACV GP1 reduced PvRBP2b₁₆₁₋₁₄₅₄ binding to reticulocytes, albeit at a lower level of inhibition as

compared with the addition of anti-TfR1 mAb MEM-189. This inhibition was specific; PfRh4 binding was unaffected by addition of MACV GP1 or MEM-189 but clearly reduced with the addition of CCP 1–3 (Fig. 1E). The addition of MACV GP1 inhibited PvRBP2b-TfR1 complex formation and reduced the FRET signal to a similar extent as unlabeled PvRBP2b₁₆₁₋₁₄₅₄, whereas addition of CCP 1–3 had negligible effect (Fig. 1F). These results indicate that MACV GP1 and PvRBP2b₁₆₁₋₁₄₅₄ bind to an overlapping site on TfR1.

Crystal structure of the N-terminal domain of PvRBP2b

PvRBP2b is a 326-kDa protein with a putative red blood cell-binding domain and a C-terminal transmembrane region (Fig. 2). We determined the crystal structure of the N-terminal domain of PvRBP2b (residues 169 to 470; PvRBP2b₁₆₉₋₄₇₀),

refined to 1.71-Å resolution (Fig. 2A; fig. S3, A to D: and table S1). The surface of the domain is mostly positively charged (Fig. 2B). It is predominantly an α -helical protein, comprising 10 α -helices and two very short antiparallel β-sheets, each comprising two β -strands. The crystal structure of PvRBP2b₁₆₉₋₄₇₀ has two disulfide bonds: one between $\rm Cys^{312}$ and $\rm Cys^{316}$ and the other between Cys²⁴⁰ and Cys²⁸⁴. This structure closely resembles the homologous domain of PvRBP2a and PfRh5, with a root mean square deviation of 1.7 and 3.7 Å over 268 and 225 aligned Cα atoms, respectively (Fig. 2C and fig. S4) (18, 33, 34). The theoretical x-ray solution scattering pattern calculated from the PvRBP2b₁₆₉₋₄₇₀ crystal structure coordinates shows excellent agreement with the experimental small-angle x-ray scattering (SAXS) data ($\gamma = 0.35$) (fig. S5, A to F, and table S2), with concordance between the crystal and solution conformations apparent from the overlay

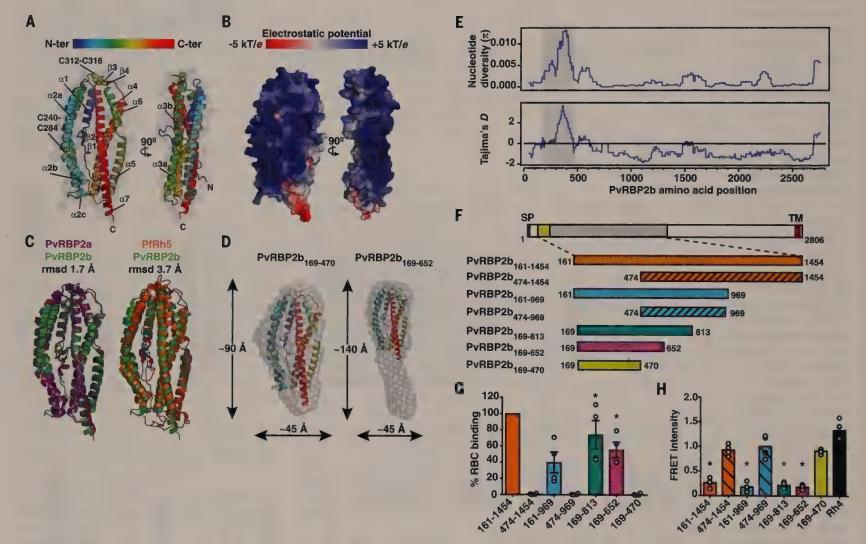


Fig. 2. Crystal structure of the N-terminal domain of PvRBP2b and its functional requirement. (**A**) Structure of the N-terminal domain of PvRBP2b from amino acid 169 to 470 shown in two orthogonal views. (**B**) Electrostatic surface potential on the PvRBP2b structure. (**C**) Superimposition of the PvRBP2b structure (green) with PvRBP2a (purple) and PfRh5 (orange). The Protein Data Bank (PDB) ID codes for PfRh5 and PvRBP2a are 4WAT and 4Z8N, respectively. (**D**) Crystal structure of the N-terminal domain superimposed with SAXS ab initio bead model of PvRBP2b_{169–470} (left) and PvRBP2b_{169–652} (right). (**E**) Sliding window analysis showing nucleotide diversity (π) values and Tajima's D statistic in PvRBP2b. The gray boxes refer to a highly polymorphic region at amino acid positions 169 to 470 that appears

to be under balancing selection. (**F**) Schematic representation of full-length PvRBP2b and recombinant protein fragments (left). Signal peptide (SP), transmembrane domain (TM), and N-terminal domain (yellow) are indicated. (**G**) PvRBP2b binding results by means of flow cytometry, in which PvRBP2b₁₆₁₋₁₄₅₄ binding was arbitrarily assigned to be 100%. (**H**) Unlabeled recombinant PvRBP2b fragments or PfRh4 were mixed at 10-fold molar excess relative to the labeled PvRBP2b₁₆₁₋₁₄₅₄–TfR1 FRET pair. The FRET intensity was relative to buffer control. For (G) and (H), mean \pm SEM, n=4 biological replicates; open circles represent biological replicates. The Mann-Whitney test was used to calculate the P value by using the binding of $2b_{474-1454}$ that was considered no binding. * $P \le 0.05$, ** $P \le 0.001$.

of the crystal structure and the ab initio calculated molecular envelope (Fig. 2D, left). We also obtained SAXS data for a longer fragment of PvRBP2b including residues 169 to 652 (fig. S6 and table S2). The reconstructed molecular en-

velope has a rodlike shape, with a C-terminal part forming a continuous extension of the N-terminal domain (Fig. 2D, right). SAXS data for a larger fragment of PvRBP2b encompassing residues 161 to 969 indicate that the molecule adopts an elongated, boomerang-like shape, similar to that previously reported for PvRBP2a (figs. S6, A to F, and S7, A to D, and table S2) (18).

We calculated nucleotide diversity (π) and Tajima's D within PvRBP2b using data from

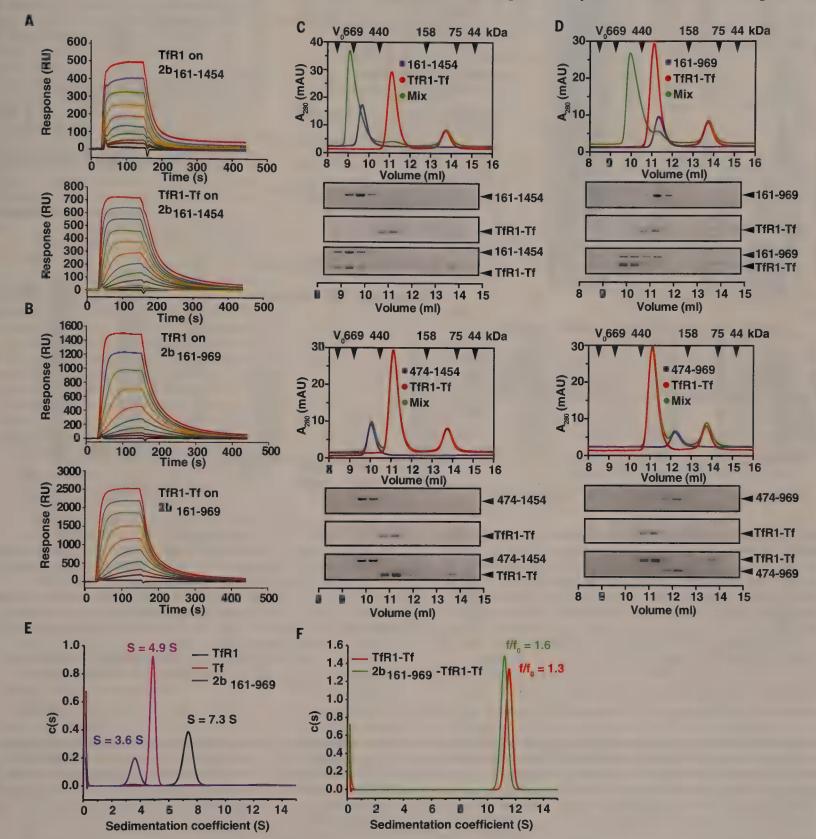


Fig. 3. PvRBP2b binds to TfR1-Tf to form a stable ternary complex. (A) PvRBP2b $_{161-1454}$ and (B) PvRBP2b $_{161-969}$ were coupled covalently to a biosensor chip to probe binding of TfR1 (concentration range assayed, 2 μ M to 7.5 nM, top) and TfR1-Tf complexes [concentration range of TfR1-Tf complexes assayed, 2:4 μ M to 1.8:3.9 nM (A) and 2:4 μ M to 7.5:15 nM (B), bottom]. (C and D) Complex formation between PvRBP2b, TfR1, and Tf analyzed by means of analytical SEC. PvRBP2b-TfR1-Tf ternary complex can be observed for PvRBP2b $_{161-1454}$ [(C), top] and PvRBP2b $_{161-969}$ [(D), top]. Two corresponding truncations of the N-terminal domain, PvRBP2b $_{474-1454}$

[(C), bottom] and PvRBP2b_{474–969} [(D), bottom], do not interact with the TfR1-Tf binary complex. The exclusion volume (V_0) of the columns and the elution volumes of selected marker proteins are indicated with black arrowheads. (Bottom) Coomassie blue–stained SDS-PAGE gels of the fractions obtained from SEC. (**E** and **F**) Continuous sedimentation coefficient distributions derived from fitting sedimentation velocity data to a c(s) sedimentation model. (E) c(s) distributions for TfR1 (black line), Tf (magenta line), and PvRBP2b_{161–969} (blue line). (F) c(s) distributions for the TfR1-Tf complex (red line) and PvRBP2b_{161–969}—TfR1-Tf complex (green line).

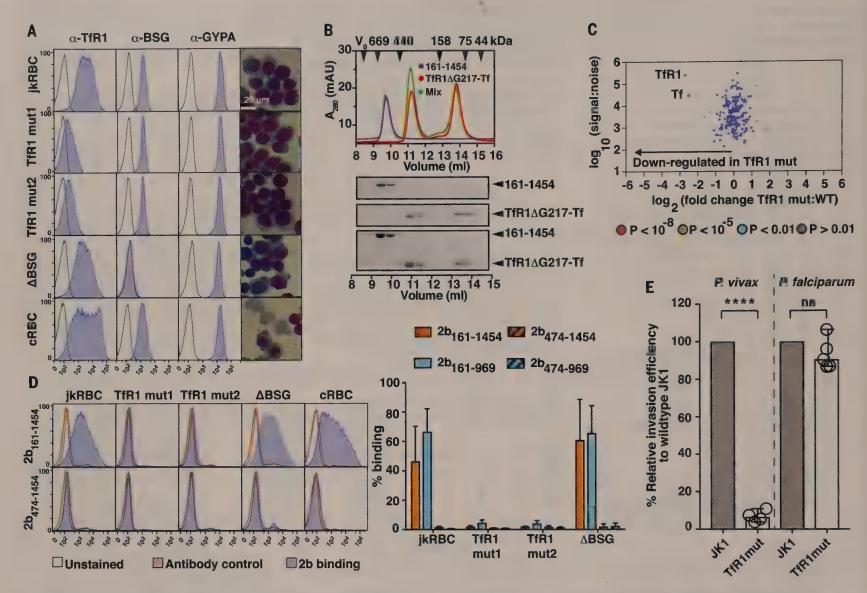


Fig. 4. Deletions in *TFRC* **reduce TfR1 surface expression, abolish PvREP2h binding, and inhibit** *P. vivax* **invasion.** (**A**) Expression of TfR1, BSG, and GypA on the surface of jkRBCs, TfR1 mutants, ΔBSG null, and cultured erythrocytes (cRBCs) as measured with flow cytometry. (Right) Cytospin analysis of cells stained with May-Grünwald Giemsa staining technique. (**B**) TfR1 Δ G217 mutation in TfR1 abrogates PvRBP2b binding as observed by using analytical SEC. (**C**) Quantitative surface proteomics demonstrate specific reduction in TfR1 protein levels in TfR1 mutants compared with wild-type jkRBCs. Levels of Tf, the binding partner for TfR1, are similarly reduced. Significance A was used to estimate *P* values, and a minimum of two peptides were required for protein

quantitation. (**D**) Binding of recombinant PvRBP2b fragments to jkRBCs, TfR1 mutants, Δ BSG, and cRBCs are shown in blue. Negative controls of unstained cells and isotype control stained cells are shown in the gray and orange lines, respectively. (Right) Compilation of results from PvRBP2b fragment binding to jkRBCs, TfR1 mutants, Δ BSG, and cRBCs. Mean \pm SEM, n=3 biological replicates. (**E**) Comparison of invasion efficiency between jkRBCs and TfR1 mutant cell lines with either *P. vivax* or *P. falciparum*. The data shown are averages and SEM from between four to five biological replicates shown as open circles. *P* value was calculated by using a paired, two-tailed t test. **** $P \leq 0.0001$; ns, nonsignificant.

the MalariaGEN *P. vivax* Genome Variation project (35). There was a peak in both metrics between amino acid positions 169 and 470, suggesting balancing selection within the N-terminal domain (Fig. 2E). Such signatures of balancing selection are often associated with genes or proteins expressed on the surface of merozoites and are likely due to interaction with the immune system.

To determine the importance of the N-terminal domain for PvRBP2b function, we generated a series of purified recombinant PvRBP2b protein fragments (Fig. 2F and tables S3 and S4). All proteins were soluble and properly folded as indicated by high α -helical content in CD spectra, which is in agreement with the secondary structure predictions (fig. S8, A to D). We observed

that all fragments with the N-terminal domain bound reticulocytes (PvRBP2b₁₆₁₋₁₄₅₄, PvRBP2b₁₆₁₋₉₆₉, PvRBP2b₁₆₉₋₈₁₃, and PvRBP2b₁₆₉₋₆₅₂), whereas their corresponding fragments without the domain did not (PvRBP2b474-1454 and PvRBP2b474-969) (Fig. 2G). However, the isolated N-terminal domain PvRBP2b₁₆₉₋₄₇₀ was unable to bind reticulocytes on its own (Fig. 2G), indicating that this fragment of PvRBP2b is necessary but not sufficient for reticulocyte binding. The shortest PvRBP2b fragment that showed binding to reticulocytes encompasses residues 169 to 652 (Fig. 2G). Our FRET-based assay showed that unlabeled recombinant fragments that bind reticulocytes inhibited PvRBP2b-TfR1 complex formation, whereas recombinant fragments that did not bind reticulocytes

had a negligible effect (Fig. 2H). Collectively, our structural and functional analyses indicate that the N-terminal domain is necessary for binding but requires the presence of the elongated C-terminal fragment to form a fully functional binding site.

PvRBP2b, TfR1, and Tf form a stable complex at nanomolar concentrations

Using surface plasmon resonance, we found that PvRBP2b₁₆₁₋₁₄₅₄ interacts with TfR1 alone or with the binary complex of TfR1-Tf (Fig. 3A, top and bottom, respectively). We also observed similar results for the PvRBP2b₁₆₁₋₉₆₉ fragment with TfR1 and TfR1-Tf (Fig. 3B, top and bottom, respectively). These results indicate that Tf was

not required for the PvRBP2b-TfR1 complex formation because the addition of Tf resulted in similar binding responses than for TfR1 alone. We analyzed a PvRBP2b, TfR1, and Tf ternary complex using analytical size exclusion chromatography (SEC) and used SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses to confirm comigration of complex components. The ternary complex was detected for PvRBP2b₁₆₁₋₁₄₅₄ and PvRBP2b₁₆₁₋₉₆₉ (Fig. 3, C and D, top, respectively, and table S5). By contrast, their corresponding fragments without the N-terminal domain (PvRBP2b₄₇₄₋₁₄₅₄ and PvRBP2b₄₇₄₋₉₆₉) did not form any observable ternary complexes (Fig. 3, C and D, bottom). The interaction between PvRBP2b and TfR1-Tf binary complex is similar in the presence of either the irondepleted or iron-loaded form of human transferrin (fig. S2C). Furthermore, the homologous member of the same protein family, PvRBP2a, did not form a ternary complex with TfR1-Tf (fig. S2D).

Sedimentation velocity analyses of TfR1, Tf, and PvRBP2b₁₆₁₋₉₆₉ indicated that the isolated proteins are homogenous, with weight-average sedimentation coefficients of 7.3, 4.9, and 3.6 S, respectively (Fig. 3E). These values are consistent with a stable dimer of TfR1 and monomeric forms of both Tf and PvRBP2b₁₆₁₋₉₆₉. The empirically fitted shape parameter value (frictional ratio) calculated for PvRBP2b₁₆₁₋₉₆₉ was ~1.8, which is consistent with a highly elongated structure in solution. Mixtures of TfR1-Tf and PvRBP2b₁₆₁₋₉₆₉-TfRI-Tf yielded single symmetrical peaks with weight-average sedimentation coefficients of 11.5 and 11.2 S, respectively, with no peaks observed for the individual components in these samples (Fig. 3F and fig. S9). These results indicate that Tf and TfR1 form a stable binary complex in solution and that PvRBP2b₁₆₁₋₉₆₉ binds to this binary complex. The frictional ratio (f/f_0) for the ternary PvRBP2b₁₆₁₋₉₆₉-TfR1-Tf was higher than for the binary TfR1-Tf complex, resulting in a reduction in the sedimentation coefficient on formation of the ternary complex and indicating that it has an elongated structure in solution.

Deletions in TfR1 generated via CRISPR/Cas9 abolishes PvRBP2b binding and *P. vivax* invasion

To investigate whether loss of TfR1 surface expression on red blood cells would affect PvRBP2b protein binding, we attempted to generate a knockout of the TFRC gene using CRISPR/Cas9 genome editing of the JK-1 erythroleukemia cell line. We obtained single-cell clones that displayed reduced expression of TfR1 and validated the mutation in two independent clones (TfR1 mut1 and TfR1 mut2) (fig. S10, A to C). Both clones contained an identical -3-bp deletion that resulted in the loss of amino acid Gly²¹⁷ in the TfR1 apical domain but left the rest of the protein in-frame. TfR1 mut1 was homozygous for this deletion, whereas TfR1 mut2 has a -3-bp deletion, as described above, on one allele and a -11-bp deletion on the other allele, the latter leading to a premature stop codon. Deletion of TFRC in a mouse model is embryonic lethal and leads to severe

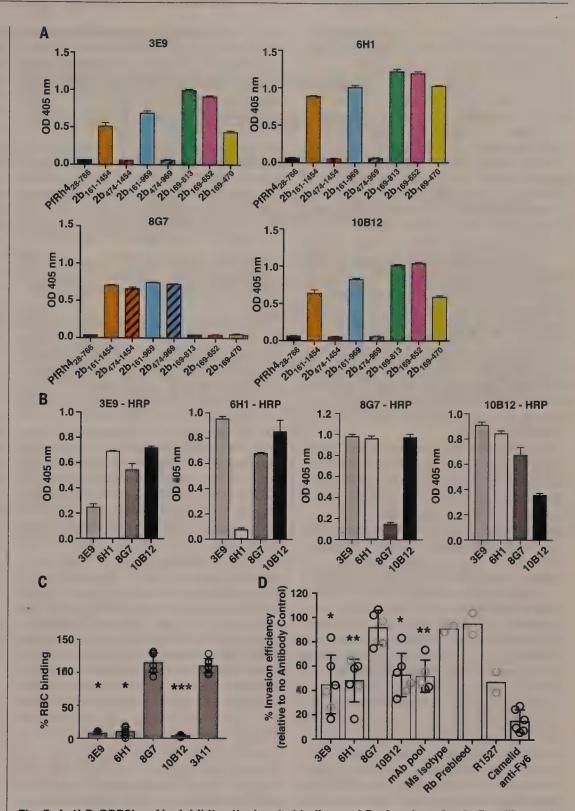


Fig. 5. Anti-PvRBP2b mAbs inhibit reticulocyte binding and P. vivax invasion In Brazilian and Thai clinical isolates. (A) ELISA plates were coated with equimolar concentrations of each recombinant fragment, and detection with anti-PvRBP2b mAbs 3E9, 6H1, 8G7, and 10B12 are shown. (B) Competition ELISA by using immobilized PvRBP2b incubated with unconjugated anti-2b mAbs (x axis) and detected with 3E9-HRP, 6H1-HRP, 8G7-HRP, and 10B12-HRP as indicated. For (A) and (B), error bars represent range showing the variability of duplicate measures. (C) PvRBP2b₁₆₁₋₁₄₅₄ binding in the presence of anti-PvRBP2b mAbs 3E9, 6H1, 8G7, and 10B12 was analyzed by means of flow cytometry. Normalized binding results in which PvRBP2b₁₆₁₋₁₄₅₄ binding in the absence of mAbs was arbitrarily assigned to be 100%. The anti-PvRBP2a mAb 3A11 was used as a negative antibody control. Mean \pm SEM, n=5biological replicates, open circles represent biological replicates. The Kruskal-Wallis test was used to calculate the P value by using 8G7 binding as no inhibition. * $P \le 0.05$, *** $P \le 0.0001$. (D) Invasion of P. vivax in Brazilian (blue open circles) and Thai (black open circles) clinical isolates in the presence of anti-PvRBP2b 3E9, 6H1, 8G7, and 10B12, pooled mAbs (each mAb at one-third of final concentration), mouse isotype control, purified rabbit prebleed IgG, purified total IgG of anti-PvRBP2b polyclonal antibody R1527, and camelid anti-Fy6 mAb. Antibodies were added in concentrations from 25 to 125 µg/ml, except for the camelid anti-Fy6 mAb, which was used at 25 μ g/ml. Mean \pm SD, n = 2 to 6 biological replicates; open circles represent biological replicates. For experiments with n > 2 biological replicates, we used the Kolmogorov-Smirnov test to compare 8G7 with 3E9, 6H1, and 10B12. * $P \le 0.05$, ** $P \le 0.001$.

disruption of erythropoiesis (36), suggesting that complete deletion of *TFRC* in erythroid-lineage cells, such as JK-1, may not be possible.

Differentiated polychromatic JK-1 cells (termed jkRBCs) express surface proteins (including TfR1) at levels comparable with those of differentiated CD34⁺ bone marrow-derived cultured red blood cells (cRBCs) (37). The jkRBCs, cRBCs, and differentiated jkRBCs with a knockout within the basigin receptor (ABSG) show normal levels of TfR1, whereas TfR1 mutant clones displayed an intermediate level of TfR1 surface staining, with a panel of anti-TfR1 mAbs (Fig. 4A and fig. S11). Levels of glycophorin A (GypA) and basigin (BSG) on these TfR1 mutant clones were similar to all control cells, showing that only TfR1 surface expression is affected on these cells (Fig. 4A). To determine whether deletion of Gly²¹⁷ affects PvRBP2b binding, we generated a recombinant TfR1 protein that lacks this amino acid (TfR1∆G217). Using SEC, we show that although TfR1∆G217 was still able to bind Tf, its binding to PvRBP2b was completely abolished (Fig. 4B). Gly²¹⁷, which resides on the lateral surface of the TfR1 apical domain, is close to the MACV GP1 interaction surface (fig. S10D) (31).

To confirm that the mutation in *TFRC* did not result in changes in expression of other red blood cell proteins, we compared the abundance of cell surface proteins between wild-type jkRBCs and the two TfR1 mutants using tandem mass tag-based quantitative surface proteomics (Fig. 4C). Out of 237 quantified surface proteins, only TfR1 and Tf were significantly modified, confirming the specificity of the *TFRC* mutations.

We next wanted to determine whether PvRBP2b binding was affected in the TfR1 mutant clones. PvRBP2b₁₆₁₋₁₄₅₄ and PvRBP2b₁₆₁₋₉₆₉ bound jkRBCs and cRBCs, whereas recombinant fragments PvRBP2b₄₇₄₋₁₄₅₄ and PvRBP2b₄₇₄₋₉₆₉ that lacked the N-terminal domain did not (Fig. 4D). By contrast, we did not detect any PvRBP2b₁₆₁₋₁₄₅₄ and PvRBP2b₁₆₁₋₉₆₉ binding to TfR1 mutant cells. This abolition of binding was specific to deletions in TFRC because PvRBP2b binding was unaffected on \triangle BSG null cells or on cRBCs (Fig. 4D, right). We also compared the invasion efficiency between jkRBCs and TfR1 mutant cell lines with either Brazilian P. vivax isolates or P. falciparum 3D7 (fig. S10E). A significant (>10-fold) reduction in invasion efficiency was observed in the TfR1 mutant line compared with the jkRBCs line with P. vivax, whereas no significant difference was observed with P. falciparum (Fig. 4E). These results validate TfR1 as the cognate receptor for PvRBP2b and that TfR1 is an essential host factor for P. vivax invasion.

Antibodies to PvRBF2b block reticulocyte binding and P. vivax invasion

To examine whether PvRBP2b antibodies could inhibit *P. vivax* invasion, we raised mouse monoclonal antibodies to PvRBP2b₁₆₁₋₁₄₅₄ and obtained four mAbs. 3E9, 6H1, and 10B12 bound epitopes within the N-terminal domain present in PvRBP2b₁₆₉₋₄₇₀ with high affinities (Fig. 5A; fig. S12, A to C; and table S6), whereas mAb 8G7

recognized an epitope outside the N-terminal domain within amino acids 813 to 969 (Fig. 5A). Competition enzyme-linked immunosorbent assay (ELISA) experiments using mAbs directly conjugated to horseradish peroxidase (HRP) show that each mAb only competed with itself for binding to PvRBP2b, showing that 3E9, 6H1, and 10B12 bind to distinct epitopes in the N-terminal domain (Fig. 5B). Neither polyclonal nor monoclonal antibodies to PvRBP2b recognize recombinant PfRh4 and five other recombinant PvRBPs, indicating that these antibodies are specific to PvRBP2b (fig. S12B) (19). Using flow cytometry, we show that addition of anti-PvRBP2b mAbs 3E9, 6H1, and 10B12 abolished the PvRBP2b₁₆₁₋₁₄₅₄ binding to reticulocytes, whereas anti-PvRBP2b mAb 8G7 and anti-PvRBP2a mAb 3A11 had no effect (Fig. 5C).

We tested the ability of the antibodies to PvRBP2b to inhibit P. vivax invasion into human reticulocytes, using a short-term P. vivax ex vivo assay with Brazilian and Thai clinical isolates (Fig. 5D, blue and black open circles, respectively). As a control, we used a camelid antibody to Fy6, which is a single monovalent VHH domain (15 kDa) (38, 39) that targets a surface-exposed epitope on DARC and blocks its interaction with PvDBP. The addition of the 25 µg/ml camelid antibody to Fy6 in ex vivo assays by using Thai isolates resulted in 85% inhibition of P. vivax invasion (Fig. 5D). Using four Thai isolates, the addition of inhibitory anti-PvRBP mAbs 3E9, 6H1, and 10B12 at 25 µg/ml resulted in 49, 45, and 42% inhibition of P. vivax invasion, respectively. To determine whether inhibition could be improved by increasing the concentration of anti-PvRBP2b mAbs to match the molarity and valency of the single VHH domain, we used 125 µg/ml of inhibitory anti-PvRBP2b mAbs. Under these conditions, we tested the invasion efficiency of two Brazilian isolates. We observed that addition of inhibitory anti-PvRBP mAbs 3E9, 6H1, and 10B12 at 125 μ g/ml resulted in 68, 45, and 57% inhibition of P. vivax invasion in Brazilian isolates, respectively (Fig. 5D). To enable quantitative analyses of the ex vivo assays, we combined our initial results of Thai and Brazilian isolates at their respective mAb concentrations. Increased concentration of the inhibitory anti-PvRBP2b mAbs resulted in an equivalent or small increase in inhibition of P. vivax invasion (Thai at 25 µg/ml versus Brazilian at 125 µg/ml). Thus, our combined sample set underestimates the level of inhibition for antibody concentrations of 125 µg/ml. As additional controls, we included the noninhibitory anti-PvRBP2b mAb 8G7 and an immunoglobulin G1 (IgG1) mouse isotype control, which displayed only 8 and 9% inhibition of P. vivax invasion, respectively (Fig. 5D). These results show that addition of anti-PvRBP2b inhibitory mAbs 3E9, 6H1, and 10B12 resulted in significant reduction of P. vivax invasion compared with the noninhibitory anti-PvRBP2b mAb 8G7 (Fig. 5D).

We show that the inhibitory anti-PvRBP2b mAbs target a domain that appears to be under balancing selection (Figs. 2E and 5A), which may result in differences in inhibition between clinical

isolates owing to the presence of polymorphic epitopes. To circumvent inter-isolate differences, we further tested the combination of all three inhibitory mAbs, 3E9, 6H1, and 10B12 pooled together (mAb pool) and polyclonal antibodies to PvRBP2b. The mAb pool resulted in significant 48% reduction in P. vivax invasion in both Thai and Brazilian isolates compared with that of anti-PvRBP2b mAb 8G7 (Fig. 5D). Addition of purified total IgG of polyclonal antibodies to PvRBP2b R1527 resulted in 53% reduction in P. vivax invasion, whereas the rabbit prebleed IgG showed only 5% inhibition (Fig. 5D). A previous study using rabbit antibodies to PvDBP shows that P. vivax invasion was reduced up to 64% (40), a level of inhibition comparable with what has been observed with our antibodies to PvRBP2b (Fig. 5D). These results show that anti-PvRBP2b mAbs that block binding to reticulocytes also inhibit P. vivax invasion and highlight the important role of the PvRBP2b-TfR1 invasion pathway in P. vivax field isolates.

Our results reveal a stable interaction between PvRBP2b and TfR1 and that antibodies to PvRBP2b that block binding to reticulocytes also inhibit P. vivax invasion into human reticulocytes. P. vivax invasion is significantly inhibited in the presence of TfR1 mutant cells, showing that TfR1 is a critical host factor for entry into reticulocytes. We propose that the PvRBP2b-TfR1 interaction is important for the initial recognition of the target reticulocyte cells, which results in the commitment of P. vivax parasites for reticulocyte invasion and the subsequent engagement of PvDBP-DARC in tight junction formation, leading to the successful completion of the invasion process. Identification of the molecular entities required for P. vivax invasion offer the possibility to target multiple invasion pathways for synergistic inhibition of P. vivax blood stage infection.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/48/suppl/DC1 Materials and Methods Figs. S1 to S12 Tables S1 to S6 References (41–83) Data Set S1

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COMPARATIVE GENOMICS

Rapid genome shrinkage in a self-fertile nematode reveals sperm competition proteins

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To reveal impacts of sexual mode on genome content, we compared chromosome-scale assemblies of the outcrossing nematode *Caenorhabditis nigoni* to its self-fertile sibling species, *C. briggsae*. *C. nigoni*'s genome resembles that of outcrossing relatives but encodes 31% more protein-coding genes than *C. briggsae*. *C. nigoni* genes lacking *C. briggsae* orthologs were disproportionately small and male-biased in expression. These include the *male secreted short* (*mss*) gene family, which encodes sperm surface glycoproteins conserved only in outcrossing species. Sperm from *mss*-null males of outcrossing *C. remanei* failed to compete with wild-type sperm, despite normal fertility in noncompetitive mating. Restoring *mss* to *C. briggsae* males was sufficient to enhance sperm competitiveness. Thus, sex has a pervasive influence on genome content that can be used to identify sperm competition factors.

ex between individuals is nearly ubiquitous in eukaryotic life (1). However, in multicellular organisms, the costs of sex and scarcity of mates sometimes favor the evolution of uniparental reproduction through asexual parthenogenesis or self-fertilization (2). Such changes in sexual reproduction have consequences for both sexual traits and genome content. Comparative genomics using closely related species with different modes of sexual reproduction can reveal sex-related factors that might otherwise remain cryptic. In the nematode species C. elegans, C. briggsae, and C. tropicalis, animals with two X chromosomes that would normally be female have evolved into self-fertilizing hermaphrodites (Fig. 1A) (3). Nearly all progeny of these selfing XX hermaphrodites are themselves XX. Rare haplo-X (XO) male progeny experience weaker sexual selection than males from outcrossing species, exhibit atrophy of traits required for efficient mating (4-7), and are hypersensitive to pheromone-induced mortality (8). Sexually antagonistic sperm-female interactions have also been relaxed in self-fertile Caenorhabditis (9).

Self-fertile *Caenorhabditis* have smaller genomes and transcriptomes than outcrossing *Caenorhabditis* (10, 11), as also observed in the selfing plant *Arabidopsis thaliana* (12). However, comparisons of self-fertilizing to outcrossing *Caenorhabditis* have involved species ■ divergent at the nucle-

otide level as humans are from mice (10, 13), so it remains unclear how quickly genomic shrinkage occurs. We hypothesized a direct link between the degradation of sexual traits and genome contraction in selfing species. Here, we describe genomic resources and functional experiments that confirm its existence.

Comparison of *C. nigoni* and *C. briggsae* genomes

Of the ~50 known Caenorhabditis species, the most closely related pair with different sexual modes are the outcrossing C. nigoni and the selfing C. briggsae (14-16). They remain partially interfertile, yet they have numerous genetic and reproductive incompatibilities (9, 15, 17-19). To compare their genomes, we assembled the C. nigoni genome from 20-kb Pacific Biosciences (PacBio) and Illumina short-read libraries (table S1) (20). The final C. nigoni chromosome-scale genome assembly totaled 129 Mb with a size-weighted median (N50) contig length of 3.3 Mb; it was estimated as 99.6% complete (21). The genome was 19% larger than that of C. briggsae (108 Mb) but was similar in size to genomes of the more distantly related outcrossing species C. remanei, C. sinica, C. brenneri, and C. japonica, which range from 131 to 135 Mb (Fig. 1A) (10). Therefore, larger genome sizes were probably the ancestral condition, and genomic shrinkage occurred in the C. briggsae lineage after it diverged from C. nigoni. More than 90% (118 Mb) of the assembly can be aligned to the chromosomes of C. briggsae without large translocations or inversions, despite megabase-sized contigs (fig. S1). Thus, the two genomes are essentially colinear but differ in many small speciesspecific segments. C. nigoni's six chromosomes are 6.6 to 16.6% larger than their C. briggsae homologs (table S2).

We used whole-genome alignment to identify species-specific genomic segments (20). In

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C. nigoni, 47.7 Mb (36.9%) did not align with C. briggsae, and C. briggsae had 27.7 Mb (25.6% of 108.4 Mb) that did not align with C. nigoni. This 20.0-Mb difference accounted for 95% of the difference in genome sizes. Nonalignable genomic regions were concentrated on the distal arms of all six holocentric chromosomes, where small inversions and repetitive sequences were abundant and gene densities were low (Fig. 1B). These regions were mostly small (median ~500 base pairs; Fig. 2A), but larger (1 to 65 kb) insertions or deletions accounted for 17 Mb (81%) of the genome size difference (Fig. 2B). In both assemblies, nonalignable sequences were most

common in intergenic regions and introns (fig. S2). *C. nigoni* harbored 5.4 Mb more species-specific protein-coding sequences than *C. briggsae*, consistent with a net loss of genes in *C. briggsae* (see below). For orthologous genes in both species, exon lengths were highly correlated (Fig. 2C and table S3). In contrast, ortholog intron content was weakly correlated and was significantly larger in *C. briggsae*. Because both genomes had similar repetitive DNA fractions (*C. nigoni* 27% versus *C. briggsae* 25%), disproportionate loss of repetitive sequences (seen in plants) did not contribute to different genome sizes (table S1) (10, 12, 22).

Impact of genome shrinkage on C. briggsae gene content

We predicted 29,167 protein-coding genes for $C.\ nigoni$ (table S4), with 88.9% (25,929) being expressed in adults [\geq 0.1 transcripts per million (TPM)]. By equivalent methods, we predicted 22,313 genes in $C.\ briggsae$ (20), 23.5% less than $C.\ nigoni$. The published gene annotations for $C.\ briggsae$ (23) were even fewer (21,814 genes).

This 6854-gene difference could have several causes, including gene family contraction and loss of sequence classes in *C. briggsae*, as well as *C. nigoni*-biased gain of novel sequences. We compared genes of *C. briggsae* and *C. nigoni* to

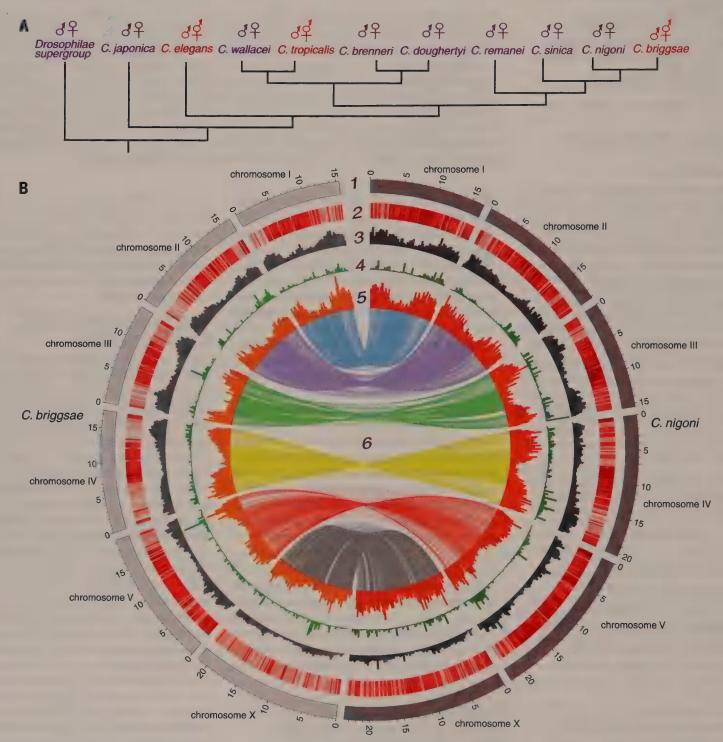


Fig. 1. The phylogenetic relationship of *Caenorhabditis* and comparison of the *C. nigoni* and *C. briggsae* genome assemblies.

(A) Phylogeny of Elegans supergroup *Caenorhabditis* [adapted from (3)] with outcrossing species producing XX females indicated in blue, and self-fertile lineages with XX hermaphrodites indicated in red.

(B) Chromosomal alignments and genomic features over 200-kb

chromosomal intervals. Tracks from outside to inside: **1**, positions (in Mb) of the six chromosomes of *C. nigoni* and *C. briggsae*; **2**, gene density heat map (darker shade indicates higher density); **3**, repeat frequency; **4**, inversion frequencies; **5**, percentage of sequence lacking homology in the other assembly (representing either deletions or species-specific gains); **6**, DNA sequence synteny.

genes of the outgroups C. remanei, C. brenneri, and C. elegans (20). In C. nigoni, 24,341 genes (83.5%) were orthologous to 21,124 C. briggsae genes, reflecting larger multigene families in C. nigoni versus C. briggsae (Fig. 3A and table S4) (24). Another 2949 C. nigoni genes without C. briggsae orthologs (10.1%) represent losses in C. briggsae based on homologs in Caenorhabditis outgroups (fig. S3). Finally, 1877 C. nigoni genes (6.4%) lacked homologs entirely and were classed as orphans. These genes could be exceptionally divergent, recently arisen in C. nigoni, or arisen shortly before the C. nigoni-C. briggsae split but then lost in C. briggsae. Overall, gene loss in C. briggsae appears to be the primary driver of the gene number difference.

To characterize genes lost in *C. briggsae*, we first compared Pfam protein domains encoded by *C. nigoni* versus *C. briggsae*. We found 26 Pfam domains that were overrepresented in *C. nigoni* (fig. S4 and table S5); of these, seven were consistently overrepresented in outcrossing *C. nigoni*, *C. remanei*, and *C. brenneri* relative to the selfing species *C. briggsae* and *C. elegans*. Three of these domains (F-box, FBA_2/F-box associated, and BTB)

are predicted to mediate protein-protein interactions. Male-female *Caenorhabditis* had 272 to 1074 genes in these families, whereas hermaphroditic *Caenorhabditis* had only 101 to 258 genes per family. Two other domains (Peptidase_A17 and DNA_pol_B_2) are associated with repetitive DNA. The final two overrepresented domains were Asp_protease_2 (possibly associated with retroelements) and DUF3557 (a nematode-specific domain, currently of unknown function). One overrepresented domain specific to *C. nigoni* was zf.RING2_finger; the RING domain gene *spe-42* is important for sperm-egg interactions in *C. elegans* (25).

Because *C. nigoni*–specific genes might encode fast-evolving proteins that lack known domains, we compared other gene properties. We found that although genes encoding medium to large proteins (≥200 residues) are similar in frequency in both species, *C. nigoni* encodes disproportionately more small proteins (<200 residues) than *C. briggsae* (Fig. 3B and table S6). As seen in other *Caenorhabditis* (11), genes with malebiased expression outnumber female-biased genes (Fig. 3C and table S7). However, even against this

background, *C. nigoni* genes without *C. briggsae* homologs are disproportionately male-biased in expression. Preferential loss of small and fast-evolving proteins thus occurred in *C. briggsae* after the adoption of selfing.

mss genes encode sperm glycoproteins lost in hermaphrodites

We hypothesized that genes with highly malebiased expression that are present in outcrossing species, but lost in selfing species, might function in sexual selection. Among such genes we identified the mss (male secreted short) family. We found one to four mss genes in the outcrossing species C. nigoni, C. sinica, C. remanei, C. brenneri, C. sp. 34, C. japonica, and C. afra, but found none in the selfing C. elegans, C. briggsae, and C. tropicalis. The mss family encodes small proteins (median 111 residues) with N-terminal signal sequences, rapidly evolving central domains with several predicted O-glycosylation sites, and Cterminal glycosylphosphatidylinositol (GPI) anchor membrane attachment signals (Fig. 3D). Enzyme treatments confirmed that MSS proteins were heavily glycosylated (fig. S5).

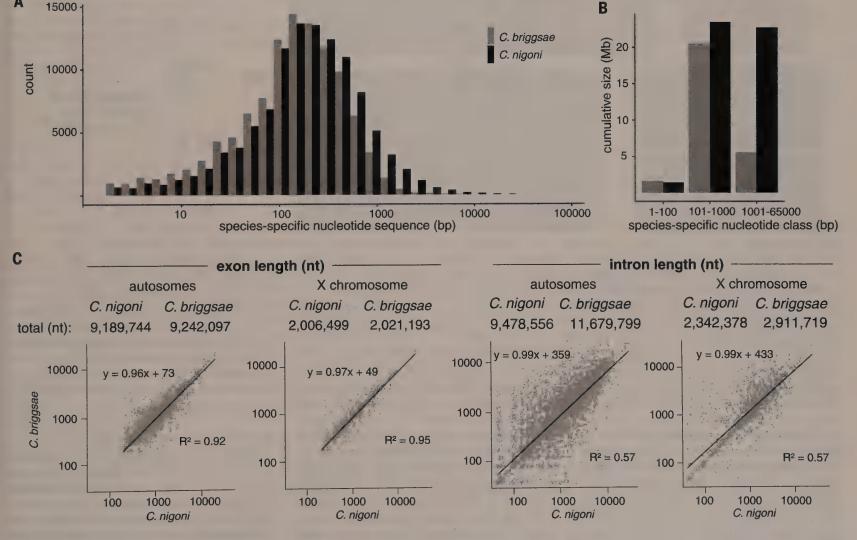


Fig. 2. Size distributions of insertion-deletion variants. (**A**) Size distribution of species-specific sequences in the *C. briggsae–C. nigoni* whole-genome alignment. Black, sequences present in *C. nigoni* alone; gray, sequences present in *C. briggsae* alone. (**B**) Contribution of different species-specific sequence types to genome size. (**C**) Regression analysis of total exon and intron lengths for 6404 one-to-one

C. briggsae–C. nigoni orthologs on autosomes and 1394 orthologs on the X chromosome. Interspecies differences were insignificant for either exon set (P = 0.378 for autosomes, P = 0.668 for X), but introns on autosomes ($P = 1.53 \times 10^{-10}$) and on the X chromosome ($P = 1.2 \times 10^{-5}$) were significantly larger in *C. briggsae* (all *P* values: Wilcoxon rank-sum test with Bonferroni correction).

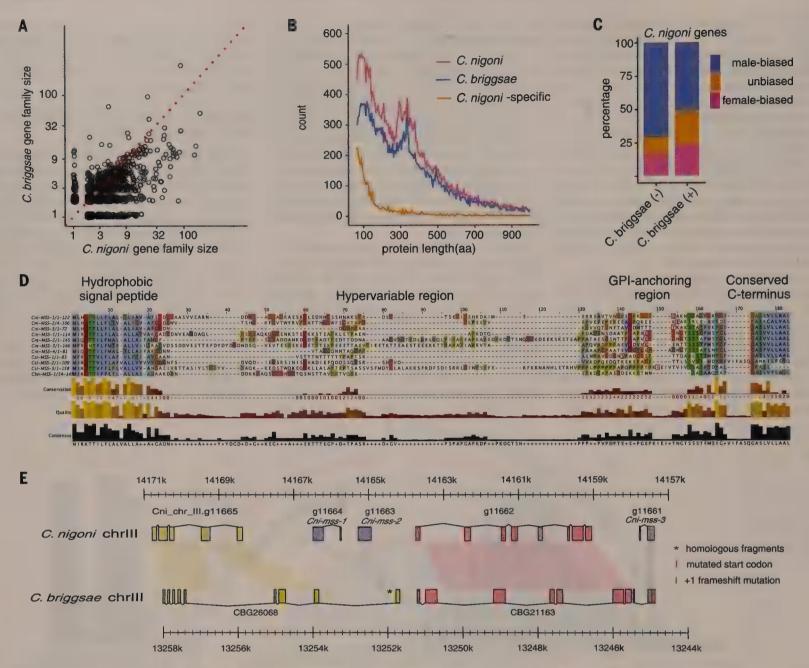


Fig. 3. Comparison of the C. nigoni and C. briggsae proteomes.

(A) Scatterplot of sizes of OrthoFinder gene families, excluding one-to-one orthologs (table S4). Of 2367 families with unequal numbers of *C. nigoni* and *C. briggsae* genes, the majority (1624) were larger in *C. nigoni* than in *C. briggsae* ($P < 2.2 \times 10^{-16}$, Wilcoxon signed-rank test). Dotted line indicates equal family sizes. (**B**) Length distributions of *C. nigoni* and *C. briggsae* proteins and of *C. nigoni* proteins that lack *C. briggsae* homologs. (**C**) For genes with sex-biased expression, male bias was seen for 50.9% of 6804 genes with *C. briggsae* homologs ["*C. briggsae* (+)"] but was significantly overrepresented (70.9%) among 605 genes lacking *C. briggsae* homologs ["*C. briggsae* (-)"; P < 0.0001, Fisher exact test; table S9]. (**D**) Alignment of predicted MSS homologs from outcrossing

C. nigoni, C. sinica, C. remanei, and C. brenneri (table S8) (20), with protein domains indicated above. Amino acid abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. (E) Comparison of mss gene regions in C. nigoni and C. briggsae. Pastel shapes connect homologous sequences. Except for Cni-mss-3, all genes are transcribed from left to right. Genes surrounding the three C. nigoni mss paralogs are conserved in C. briggsae, but only fragments and a pseudogene (Cbr-mss-3-ps) of the mss genes remain. The pseudogene has a lost start codon and a +1 frameshift. CBG26068 has a novel 3' exon derived from part of the Cni-mss-1 second exon. See fig. S7 and (20) for details.

Although we failed to detect *mss* genes in selfing species, we did discover a larger family of *mss*-related protein (*msrp*) genes, within which *mss* forms a monophyletic clade (fig. S6) (20). Notably, *msrp* genes are found both in outcrossing *Caenorhabditis* and in the hermaphroditic *C. elegans, C. briggsae*, and *C. tropicalis* (fig. S6). Like MSS proteins, MSRP proteins are small and are predicted to be secreted, O-glycosylated, and (often) GPI-anchored. Both *mss* and *msrp* genes show male-biased expression in *C. nigoni* and other species (table S8). In cases where their chromosomal loci can be identified, *mss* and

msrp genes are autosomal; this linkage fits n general pattern in heterogametic male species of male-biased genes being autosomal rather than X-chromosomal [(26) and references therein].

Because we observed *mss* genes in two *C. elegans* outgroups (*C. japonica* and *C. afra*; fig. S6 and table S8), their absence from hermaphrodites most likely reflects independent gene losses rather than phylogenetic restriction to close relatives of *C. nigoni*. Examination of the *C. briggsae* genomic region syntenic to the *C. nigoni mss* locus revealed fragments of *mss-1* and *mss-2* coding sequences and a nearly complete *mss-3* pseudo-

gene (Fig. 3E) (20). Mutations that ablate *Cbr-mss-3-ps* function in the AF16 reference strain also occur in 11 wild isolates that span the known diversity of *C. briggsae* (fig. S7) (20, 27). Orthologs of all three *C. nigoni mss* genes were therefore present in the common ancestor of *C. nigoni* and *C. briggsae* but were lost in *C. briggsae* before its global diversification.

In the outcrossing species *C. remanei*, *mss* transcripts were expressed only in adult males (Fig. 4A), with strongest expression in spermatocytes during mid-pachytene of meiosis I (Fig. 4B). To determine subcellular localization of MSS

Fig. 4. *C. remanei* MSS is a male-specific protein localized to the surface of activated sperm.

(A) Quantitative reverse transcription polymerase chain reaction (for *Cre-mss-2*) of mixed-sex populations (top) versus larval and adult sex-specific populations (bottom), showing that *mss* expression is specific to adult males. Data are means ± SEM. Female data are two to three orders of magnitude below male data.

(B) *Cre-mss-1* transcripts are detected in pachytene-stage primary spermatocytes.

(C) Dissected testis expressing

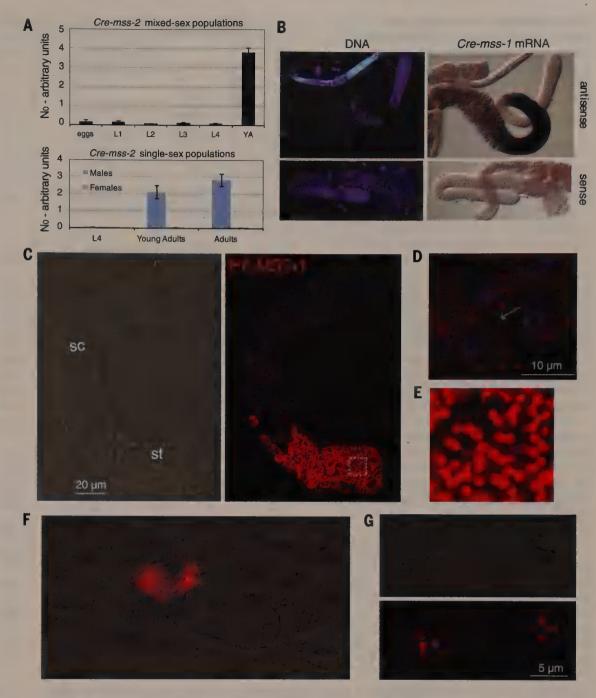
(C) Dissected testis expressing HA-tagged Cre-MSS-1, viewed with differential interference contrast (DIC, left) or anti-HA confocal fluorescence (right) microscopy. Cre-HA-MSS-1 is first detectable in spermatocytes (sc) and becomes enriched in spermatids (st). (D) Some Cre-HA-MSS-1 is localized to the plasma membrane of spermatocytes. as indicated by the arrow. Blue fluorescence: Hoechst-stained DNA. (E) Enlarged view of the boxed region in (C), showing complete restriction to membranous organelles

attached to sperm after activation and transfer to the female. (G) Cre-HA-MSS-1 sperm cells dissected from a female and stained with anti-HA immunohistochemistry, imaged with DIC (top) and confocal (bottom) microscopy. Cre-HA-MSS-1 is visible in the plasma membrane and fused MO remnants.

(MOs). (F) Cre-HA-MSS-1 remains

peptides, we used CRISPR/Cas9 editing to tag the *Cre-mss-I* gene of *C. remanei* with the hemagglutinin (HA) epitope. *Crem-MSS-1*::HA expression was first detected in large vesicles and on the plasma membrane of spermatocytes, with intensity increasing and localization restricted to secretory vesicles in mature spermatids (Fig. 4, C to E). The secretory vesicles of nematode sperm, known as membranous organelles (MOs), fuse with the plasma membrane upon ejaculation and sperm activation (28).

MSS peptides might be processed by a signal peptidase to release a soluble fragment into the MO lumen, which could then be dumped into seminal fluid upon sperm activation. However, their transient plasma membrane localization in spermatocytes and predicted C-terminal GPI attachment signals (Fig. 3D and table S8) suggested that MSS peptides might instead be attached to membranes. Consistent with this latter hypothesis, *Crem*-MSS::HA remained associated with activated sperm dissected from inseminated females (Fig. 4F). We observed staining of the plasma membrane and of MO-derived punctae



(Fig. 4G), which may be fused vesicles that remain as cup-like invaginations (29). Persistence of MSS on the surface of sperm after activation suggested that MSS acts cell-autonomously, rather than through the seminal fluid.

mss genes mediate sperm competition and affect sex ratios

Because the four C. remanei mss paralogs form a 7-kb tandem array (fig. S8A), we deleted the entire mss cluster via CRISPR/Cas9 editing. To avoid inbreeding depression associated with homozygosity of entire chromosomes (30) (fig. S8B), we generated the mss deletion in two different C. remanei strains and crossed them to create hybrid mss-null mutants. The resulting males showed no intrinsic fertility defects, as judged by overall brood size (fig. S8C). However, when competing against heterozygous mss(null/+) males, mss mutants sired fewer progeny than nonmutants in both offense (mutant male second) and defense (mutant male first) scenarios (Fig. 5, A and B). The mss family is therefore required for male sperm competitiveness in multiple mating situations, but

not for fertility itself. Sperm lacking MSS compete poorly even when the female reproductive tract is conditioned by wild-type sperm. Thus, MSS proteins probably do not function as a secreted signal, but instead act cell-autonomously.

We then introduced *mss-1* and *mss-2* genes from *C. nigoni* into *C. briggsae* via a low-copy, germline-expressed MSS transgene; this transgene was strongly expressed in *C. briggsae* males, while also being detectable in hermaphrodites (fig. S9). Remarkably, sperm from transgenic *mss(+) C. briggsae* males outcompeted those of wild-type males (Fig. 5, C and D). After *mss(+)* sperm were exhausted, however, wild-type *mss* (null) sperm were still fertilization-competent (Fig. 5, C and D). In addition, *mss(+)* males were more consistently able to suppress use of a hermaphrodite mate's self-sperm (Fig. 5E).

Because 50% of outcross progeny are male whereas selfed progeny are almost exclusively hermaphrodites, we examined the effect of transgenic *mss* on long-term sex ratios in *C. briggsae* populations. We started both wild-type and *mss*(+) *C. briggsae* populations with a 1:1 male-to-hermaphrodite

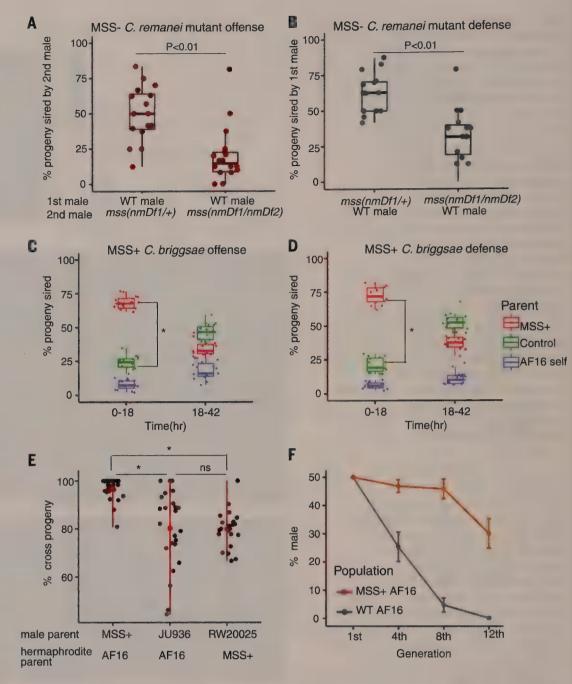
Fig. 5. mss genes are necessary for sperm competitiveness in an outcrossing species, and sufficient to enhance it in selfing species. (A) When mated after a wild-type male ("offense"), C. remanei mss (nmDf1/+) males sire more than twice the progeny of nmDf1/nmDf2 mss-null mutants (N = 16 for both). (B) When allowed to mate first ("defense"), heterozygous C. remanei mss(nmDf1/+) males have a slight advantage over wild-type males; mss-null mutants, in contrast, do not (N = 15 for both). Heterozygote success is assumed to be double the observed nmDf1 frequency in their progeny. For both defense and offense, P < 0.01(two-sample Kolmogorov-Smirnov test). (C and D) Wild-type young C. briggsae hermaphrodites were mated sequentially (4 hours each) with conspecific males carrying either a C. nigoni mss(+) transgene or a control mCherry::histone reporter (RW0025). Progeny laid 0 to 18 hours and 18 to 42 hours after the second mating were scored for green (MSS+), red (RW0025), or no (self) fluorescent markers. In both offense (C) and defense (D), MSS+ males sire several times as many progeny as control males in the first laying window. *P < 0.001. (E) MSS+ C. briggsae males suppress selfing more effectively than do control AF16 (wild-type) males. Strain JU936 is a second control strain bearing two transcriptional GFP reporters in the AF16 background. *P < 0.001 (Kolmogorov-Smirnov test); ns, not significant. (F) Male frequency in MSS+ and wild-type AF16 C. briggsae populations in which male frequency was artificially elevated to 50% at the start of the experiment. In all panels except (E), error bars denote SD.

sex ratio and examined them over time. Wild-type *C. briggsae* showed a rapid decline of males, as previously seen in *C. elegans* (7, 31). However, male frequency remained elevated in the *mss*(+) strain (Fig. 5F), only declining after 12 generations. The expression of MSS proteins was thus sufficient to shift population sex ratios toward parity.

Discussion

Comparison of the *C. nigoni* and *C. briggsae* genomes revealed that *C. briggsae* experienced rapid contraction of chromosomes and loss of protein-coding genes. However, loss of ancestral genomic content in *C. briggsae* does not fully explain their genomic divergence; the ongoing birth of novel sequences in both species, along with loss of ancestral DNA in *C. nigoni*, is also important. Net shrinkage of the *C. briggsae* genome therefore resulted from a substantial increase in the ratio of losses to gains. These losses included many coding sequences, reducing the *C. briggsae* gene count by nearly one-quarter.

Multiple observations implicate the evolution of selfing as the cause of genome shrinkage in *C. briggsae*. Reduced genome and transcriptome



sizes are observed in all three selfing Caenorhabditis species (10, 11). Continued interfertility of C. briggsae and C. nigoni (15) indicates that self-fertility and genome shrinkage evolved in quick succession. Genes with male-biased expression, such as the mss family, are disproportionately and consistently lost from selfing species (II). This suggests that genes with male reproductive functions that are either dispensable or maladaptive in the new sexual mode are purged from the genome. Finally, the net genome shrinkage we observed has been predicted to arise from a partially selfing mating system coupled with transmission distortion of autosomal deletion alleles (32, 33). Such distortion is driven by imbalanced chromatin during meiosis I of XO males, and causes preferential inheritance of shorter alleles by hermaphrodite progeny and their increased fixation in the population.

Larger autosomal deletions, influenced most by the deletion segregation distortion mechanism, are primarily responsible for the smaller genome of *C. briggsae* (Fig. 2). However, such deletions and net shrinkage were also found on the X chromosome (table S2), which should be unaffected. Moreover, orthologous genes have larger introns in C. briggsae than in C. nigoni (Fig. 2), and introns constitute a greater fraction of the C. briggsae genome (fig. S2). X-chromosomal C. briggsae introns are also larger than those of the outgroup C. remanei (10) (fig. S2C), which suggests that introns of many genes expanded in C. briggsae. Thus, additional processes must also contribute to shrinkage of the C. briggsae genome. Spontaneous short (1- to 5-nucleotide) mutations in C. elegans are biased toward insertions rather than deletions (34), although biases in formation of larger indels remain uncharacterized. Regardless, the relative rates of insertion and deletion mutations likely evolved too slowly to explain C. briggsae's reduced genome size, given its recent divergence from C. nigoni (27). Gene loss can sometimes be adaptive (35, 36) and has been proposed as a factor promoting genome shrinkage in selfing Caenorhabditis (10). Our results for the C. nigoni-C. briggsae pair support this hypothesis.

Genes encoding small proteins with male-biased expression are disproportionately lost in *C. briggsae*; here, *mss* provides an instance affecting reproduction. Unlike *comp-1*, which encodes a kinase required for male versus hermaphrodite sperm

competition in C. elegans (37) and which is conserved regardless of mating system, we found mss orthologs only in outcrossing species. In interspecies matings, sperm from males of outcrossing species rapidly invade the ovaries and body cavities of selfing hermaphrodites, sterilizing or killing them (9). This cryptic toxicity of outcrossing sperm is likely due to ongoing sexual selection in outcrossing species. Given their pronounced role in sperm competition, MSS proteins may contribute to sperm invasiveness.

How MSS improves sperm competitiveness remains unclear, but mature MSS proteins are substantially glycosylated (fig. S5). Such posttranslational modification may impose little constraint on MSS proteins, explaining how they can have weak sequence conservation yet strong functional conservation. Another poorly conserved O-glycosylated protein, the mucin PLG-1, forms a copulatory plug found in all male-female Caenorhabditis species but lost in many wild isolates of C. elegans (4). Glycoproteins form the glycocalyx coat of mammalian sperm and play important roles in fertility (38). Caenorhabditis provides a useful model for how the glycocalyx and female tissues interact and how these interactions affect sperm competition.

Independent loss of mss in the three known hermaphroditic Caenorhabditis species could reflect relaxed sexual selection coupled with mutation and drift, or it could reflect adaptive convergence. Other changes in selfing species—such as loss of plg-1 and of plep-1, which mediates reliable male discrimination between the vulva and excretory pore (4, 6)—are likely due to relaxed selection. However, restoring mss to C. briggsae enhances male fitness (Fig. 5, C and D), and mutations inactivating the Cbr-mss-3-ps pseudogene are not deletions that would be subject to loss via transmission ratio distortion (fig. S7). These findings suggest that loss of mss may instead reflect adaptive convergence, permitting protohermaphrodites to adapt to a selfing lifestyle and resolve emergent sexual conflicts related to mating (39-41). Selfing Caenorhabditis species lack inbreeding depression (42) and reproduce in spatially isolated habitats colonized by small numbers of founders (3). Reduced male mating success creates hermaphrodite-biased sex ratios (Fig. 5F), which may be adaptive under these conditions (41, 43-45). Thus, evolutionary transitions in reproductive mode may produce conditions for selection to rapidly eliminate formerly constrained reproductive genes.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/55/suppl/DC1 Materials and Methods Figs. S1 to S9 Tables S1 to S9

Data S1 to S5 References (46-119)

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REPORTS

MATERIALS SCIENCE

Hydraulically amplified self-healing electrostatic actuators with muscle-like performance

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Existing soft actuators have persistent challenges that restrain the potential of soft robotics, highlighting a need for soft transducers that are powerful, high-speed, efficient, and robust. We describe a class of soft actuators, termed hydraulically amplified self-healing electrostatic (HASEL) actuators, which harness a mechanism that couples electrostatic and hydraulic forces to achieve ■ variety of actuation modes. We introduce prototypical designs of HASEL actuators and demonstrate their robust, muscle-like performance as well as their ability to repeatedly self-heal after dielectric breakdown all using widely available materials and common fabrication techniques. A soft gripper handling delicate objects and a self-sensing artificial muscle powering a robotic arm illustrate the wide potential of HASEL actuators for next-generation soft robotic devices.

uman-made machines rely on rigid components and excel at repetitive tasks in a structured environment, whereas nature predominantly uses soft materials for creating versatile systems that conform to their

environment. This discrepancy in mechanics has inspired the field of soft robotics (1-4), which promises to transform the way we interact with machines and to enable new technologies for biomedical devices, industrial automation, and

other applications (2, 5, 6). For soft robotics to proliferate, there is a need for an artificial muscle technology that replicates the versatility, performance, and reliability of biological muscle (2).

Currently, soft robots predominantly rely on fluidic actuators (7), which can be designed to suit a variety of applications (8-10). However, fluidic actuators require a supply of pressurized gas or liquid, and fluid transport must occur through systems of channels and tubes, limiting speed and efficiency. Thermally activated artificial muscle actuators made from inexpensive polymer fibers can provide large actuation forces and work density, but these are difficult to control and have low efficiency (1.32%) (11). Electrically powered muscle-mimetic actuators, such as dielectric elastomer (DE) actuators, offer high actuation strain (>100%) and potentially high efficiency (80%) and are self-sensing (12-14). However, DE actuators are driven by high electric fields, making them prone to failure from dielectric breakdown and electrical aging (15). Fault-tolerant DE actuators have been demonstrated that rely on localized destruction of the electrodes or dielectric to isolate the location of breakdown (16, 17). Dielectric materials made of silicone sponges swollen with silicone oil (18) continued operating after dielectric failure but demonstrated actuation strains only below 5%. More important, DE actuators are difficult to scale up to deliver high forces, as large areas of dielectric are required [e.g., in stacked actuators (13)], which are much more likely to experience premature electrical failure, following the Weibull distribution for dielectric breakdown (19).

Here, we develop a class of high-performance, versatile, muscle-mimetic soft transducers, termed HASEL (hydraulically amplified self-healing electrostatic) actuators. HASEL actuators harness an electrohydraulic mechanism to activate all-softmatter hydraulic architectures, combining the versatility of soft fluidic actuators with the musclelike performance and self-sensing abilities of DE actuators. In contrast to soft fluidic actuators, where inefficiencies and losses arise from fluid transport through systems of channels, HASEL actuators generate hydraulic pressure locally via electrostatic forces acting on liquid dielectrics distributed throughout a soft structure. The use of liquid dielectrics in HASEL actuators enables self-healing with immediate recovery of functionality after numerous dielectric breakdown

To discuss fundamental physical principles, we describe one design for HASEL actuators, where an elastomeric shell is partially covered by a pair of opposing electrodes and filled with a liquid dielectric (Fig. 1A). Applying voltage induces an electric field through the liquid and elastomeric dielectric. The resulting electrostatic Maxwell stress (20) pressurizes and displaces the liquid dielectric from between the electrodes to the surrounding

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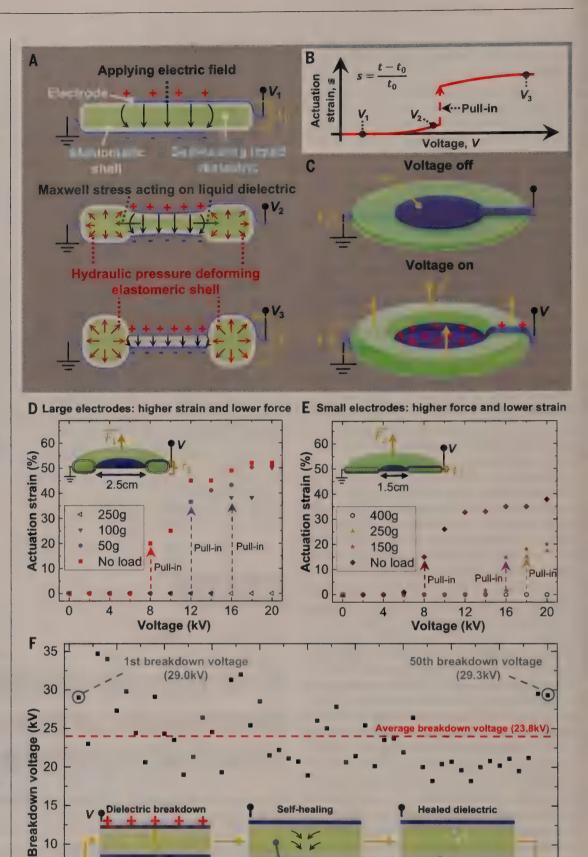


Fig. 1. Basic components and fundamental physical principles of HASEL actuators. (**A**) Schematic of a HASEL actuator shown at three different applied voltages, where $V_1 < V_2 < V_3$. (**B**) Typical actuation response of a HASEL actuator with geometry shown in (A). (**C**) The actuator deforms into a donut shape with application of voltage. This voltage-controlled deformation can be used to apply force F onto an external load. (**D** and **E**) Strain and force of actuation can be tuned by modifying the area of the electrode. The minimum electric field to trigger the pull-in transition was ~2.7 kV/mm; the maximum field applied was ~33 kV/mm. (**F**) The use of a liquid dielectric confers self-healing capabilities to HASEL actuators.

Self-healing liquid dielectric

25

Self-healing event number

30

35

40

20

15

10

45

50

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volume. As voltage increases from V_1 to V_2 , there is a small increase in actuation strain s. When voltage surpasses a threshold V_2 , the increase in electrostatic force starts to exceed the increase in mechanical restoring force, causing the electrodes to abruptly pull together (Fig. 1B)-a characteristic feature of a so-called pull-in or snap-through transition. Pull-in transitions and other nonlinear behaviors are features of soft active systems that offer opportunities to improve actuation response or functionality (21) and have been used to amplify response of fluidic (22) and DE actuators (23). After the pull-in transition (Fig. 1A), actuation strain further increases with voltage (Fig. 1B). For this design, hydraulic pressure causes the soft structure to deform into a toroidal or donut shape (Fig. 1C).

Hydraulic pressure within the elastomeric shell is coupled to Maxwell pressure, $p \propto \varepsilon E^2$, where ϵ is the dielectric permittivity of the material system and E is the applied electric field (20). Because Maxwell pressure is independent of the electrode area, actuation force and strain can be scaled by adjusting the ratio of electrode area to total area of the elastomeric shell. We fabricated two donut HASEL actuators (fig. S1) (24) that were identical except for their respective electrode diameters. The donut HASEL actuators were made from polydimethylsiloxane (PDMS: Sylgard 184, Dow Corning) as the elastomeric shell, a vegetable-based transformer oil (Envirotemp FR3, Cargill) as the liquid dielectric, and ionically conductive polyacrylamide (PAM) hydrogels as the electrodes. The actuator with larger electrodes displaced more liquid dielectric, generating a larger strain but a smaller force, because the resulting hydraulic pressure acts over a smaller area (Fig. 1D and fig. S2). Conversely, the actuator with smaller electrodes displaced less liquid dielectric, generating less strain but more force, because the resulting hydraulic pressure acts across a larger area (Fig. 1E). We tested the cycle life of a donut HASEL actuator used in Fig. 1E for more than 1 million cycles while lifting 150 g (actuation stress ~0.75 kPa) at 15% strain and noticed no perceivable loss of performance (fig. S3). We performed a full-cycle analysis of actuator efficiency using force displacement and voltage charge work-conjugate planes (fig. S4) (24). Conversion efficiency was 21%, which is comparable to typical experimental values for DE actuators; whereas DE actuators have potentially high efficiencies (80%) (12), experimentally measured efficiency ranges from 10 to 30% (25-27).

The use of liquid dielectrics enables HASEL actuators to self-heal from dielectric breakdown. In contrast to solid dielectrics, which are permanently damaged from breakdown, liquid dielectrics immediately return to an insulating state (fig. S5 and movie S1). This characteristic allowed donut HASEL actuators to self-heal from 50 dielectric breakdown events (Fig. 1F and movie S2). Although breakdown through the liquid produced gas bubbles, which have low breakdown strength, the bubbles had a limited impact on self-healing performance because they were forced away from the region of highest electric field between the electrodes (movie S2). Dielectric breakdown is

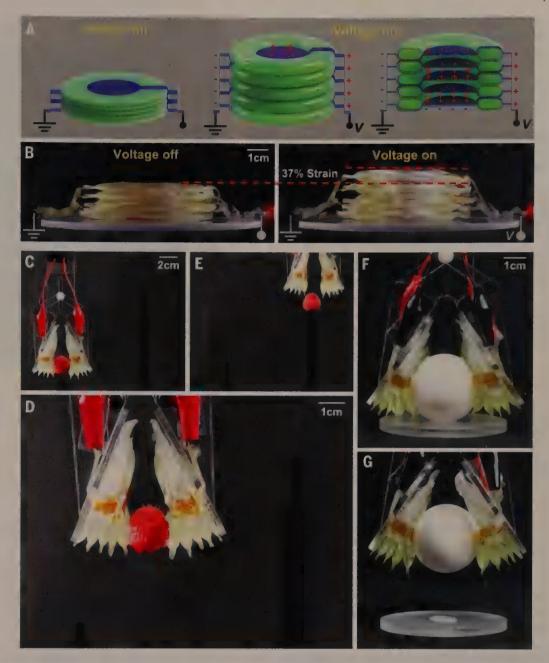


Fig. 2. Stacks of donut HASEL actuators operating as linear actuators and soft grippers. (A) Schematic depicting a stack of five donut HASEL actuators oriented such that adjacent electrodes are at the same electrical potential (cross-section view). (B) Demonstration of linear actuation with stacked-donut HASEL actuators. (C to G) A soft gripper fabricated from two modified stacks of donut HASEL actuators handled fragile objects such as a raspberry [(C) to (E)] and a raw egg [(F) and (G)].

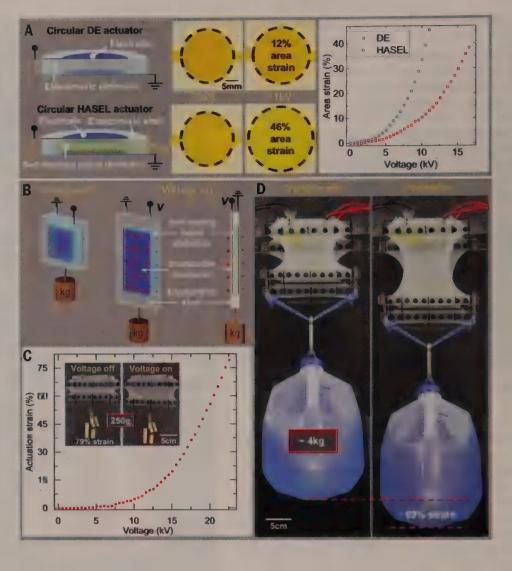
a statistical process (19), and voltage varied over 50 cycles, with several breakdown voltages exceeding the initial breakdown voltage (including the last one shown; Fig. 1F).

The ability of HASEL actuators to self-heal from electrical damage provides the means to scale up devices to produce a large actuation stroke by stacking multiple actuators (Fig. 2A). A stack of five donut HASEL actuators achieved 37% linear strain, which is comparable to linear strain achieved by biological muscle (26) and corresponds to an actuation stroke of 7 mm (Fig. 2B). Hydraulic pressure is generated locally in HASEL actuators, and liquid dielectrics are displaced over short distances, allowing for high-speed actuation. The stacked actuators readily showed large actuation response up to a frequency of 20 Hz (movie S3).

We modified two stacks of donut HASEL actuators to operate as a soft gripper, a common application for soft robotics (8, 28). Actuators within the stacks were constrained on one side to produce a tilting motion (Fig. 2, C to G, and fig. S6). When a DC voltage was applied to the stacked HASEL actuators, the device grasped delicate objects such as a raspberry (Fig. 2, C to E, and movie S4) and a raw egg (Fig. 2, F and G, and movie S4).

The geometry of HASEL actuators, like that of soft fluidic actuators, can be adapted to react with a variety of different actuation modes. For planar HASEL actuators, the electric field is applied over almost the entire region of the actuator containing liquid dielectric. Planar HASEL actuators react to application of voltage with in-plane expansion, resembling a commonly used mode of operation for DE actuators, where an elastomeric dielectric contracts in thickness and expands in area under an applied electric field. To compare the actuation response of HASEL and DE actuators, we measured area strain as a function of voltage for two circular actuators with the same

Fig. 3. Design and performance of planar HASEL actuators. (A) For a given voltage, a circular planar HASEL actuator achieves larger area strain in comparison to a circular DE actuator. (B) Schematic of a planar HASEL actuator that functions as a linear actuator. The actuator is prestretched laterally and a load is applied in the direction perpendicular to the prestretch. (C) Demonstration of linear actuation with a single-unit planar HASEL actuator. (D) HASEL actuators can be readily scaled up to exert large forces.



total dielectric thickness, t (Fig. 3A). Both were fabricated from Ecoflex 00-30 (Smooth-on); however, one-third of the thickness of the HASEL actuator was liquid dielectric, t_{liq} (24). At 11 kV, the area strain of the HASEL actuator exceeded the area strain of the DE actuator by a factor of ~4 (Fig. 3A and fig. S7). The higher actuation strain is attributed to the layer of liquid dielectric, which effectively reduces the modulus of the HASEL actuator while maintaining the high dielectric strength of the layered dielectric structure.

Linear actuation can be achieved with planar HASEL actuators by implementing a fixed prestretch in one planar direction and applying a load in the perpendicular planar direction (29). This lateral prestretch causes a preferential expansion in the direction of the load when voltage is applied (Fig. 3B). We fabricated single- and two-unit planar HASEL actuators, where a unit is defined as a discrete region of liquid dielectric (figs. S8 and S9) (24). Linear actuators were oriented vertically with the load applied in the direction of gravity, but they can be operated in any orientation as long as the liquid dielectric regions are sufficiently small to limit uneven distribution of liquid dielectric (fig. S10). A singleunit planar HASEL actuator (Fig. 3C) was activated by increasing DC voltage in discrete steps and achieved a maximum of 79% linear actuation strain under a load of 250 g (actuation stress ~32 kPa), exceeding typical values of strain observed for biological muscle (26). Soft active devices such as HASEL actuators are elastic systems that can be used near resonances to improve performance and efficiency (30)—a characteristic that could find use in legged robots that move over long distances. We found that for planar HASEL actuators, linear actuation is amplified near a resonant frequency; a single-unit actuator achieved 107% linear strain under a load of 250 g (actuation stress ~32 kPa) and a two-unit actuator achieved 124% linear strain under a load of 700 g (actuation stress ~114 kPa) (fig. S11 and movie S5). Peak specific power during contraction of the two-unit actuator was 614 W/kg; specific work during contraction was 70 J/kg (fig. S12) (24). The measured peak specific power is double that of natural muscle and comparable to values for silicone DE actuators (26). Thermally activated coiled polymer fiber actuators (49.9 kW/kg) (11) and shape-memory alloys (50 kW/kg) (11, 26) have higher peak specific power; however, their efficiency is low (<2%) (11, 26) and thermomechanical actuators are more difficult to control than electromechanical actuators. Cycle life at high mechanical output power was demonstrated with a single-unit HASEL actuator, which provided 358 W/kg average (586 W/kg peak) specific power during contraction until mechanical rupture occurred at 158,061 cycles (fig. S12D). A single unit actuator was able to operate under a large applied load of 1.5 kg [corresponding to a stress of 0.3 MPa,

near the maximum value for mammalian skeletal muscle (26)] and still achieved 16% strain (fig. S13).

Planar HASEL actuators were also able to selfheal from dielectric breakdown for at least 50 cycles, although, relative to donut HASEL actuators, gas bubbles were more easily trapped between the electrodes (fig. S14). Nonetheless, the ability of planar HASEL actuators to tolerate high electric fields applied over large areas enabled us to scale up actuation force by combining six planar HASEL actuators in parallel to lift a gallon of water (~4 kg, which corresponds to ~120 kPa) at 69% linear actuation strain (Fig. 3D and movie S6). The combination of high actuation strain and the ability to scale up for large actuation force is critical for developing high-performance soft robotic actuators for human scale devices.

Soft robotic actuators require feedback to sense and regulate position. The electrodes of a HASEL actuator form a hyperelastic capacitor with capacitance C, which is directly linked to geometry and actuation strain via $C \propto A/d$, where A is electrode area and d is the distance between electrodes. Consequently, HASEL actuators are able to selfsense deformation attributable to external forces or applied voltage. Because HASEL actuators are equivalent resistor-capacitor circuits, capacitance can be measured transiently by applying a lowamplitude AC voltage (14), then analyzing the phase and amplitude of voltage and current signals (fig. S15) (24). The low-amplitude AC signal can

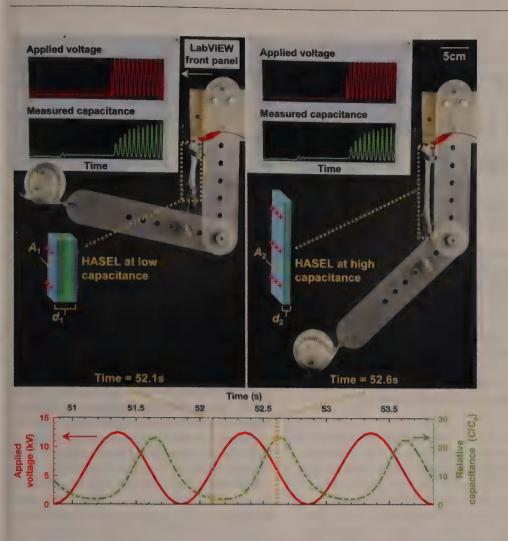


Fig. 4. A self-sensing planar HASEL actuator powering ■ robotic arm. HASEL actuators simultaneously serve as strain sensors; measured capacitance is low when the arm is fully flexed (left; screenshot of movie S7 at 52.1 s) and capacitance is high when the arm is extended (right; at 52.6 s). The bottom plot shows details of the applied voltage signal (red) and measured relative capacitance (green, dashed), C/C_0 , where C is measured capacitance and C_0 is the minimum value for capacitance. Voltage and capacitance are ~90° out of phase, which is typical for a driven damped oscillator.

be superimposed onto a high-amplitude actuation voltage signal, so only one set of electrical connections is required for both actuation and sensing. To demonstrate self-sensing actuation, we powered a robotic arm with two planar HASEL actuators combined in parallel and simultaneously measured capacitance (Fig. 4, fig. S16, and movies S7 and S8). Here, we only measured capacitance and did not attempt to control position of the robotic arm; however, capacitive self-sensing has been used for closed-loop control of DE actuators (31).

HASEL actuators rely on all-soft-matter hydraulic architectures and local generation of hydraulic pressure via electrostatic forces acting on self-healing liquid dielectrics—a recipe that combines the strengths of soft fluidic and electrostatic actuators while addressing important problems of each. The use of hydraulic principles in HASEL actuators results in the capability to scale actuation force and strain-a feature also used in other device classes such as microhydraulic systems, which are constructed from thin films and rigid substrates (32), and in hydrostatically coupled DE actuators (33), where electric fields are applied across elastomeric layers, which do not self-heal after dielectric breakdown. We have demonstrated versatile, robust, muscle-like performance of HASEL actuators made from one set of inexpensive, widely available materials and using only basic fabrication techniques. However, the thick elastomer shells (>1 mm) used in this work required high voltages to reach electric fields large enough for actuation. This need for high voltage is an existing limitation that may be addressed

by using dielectric layers with higher permittivity and by using advanced fabrication techniques to produce high-resolution dielectric structures with feature sizes on the order of 10 μ m. With a plethora of geometries, materials, and advanced fabrication strategies still unexplored, HASEL actuators offer a new platform for research and development of muscle-mimetic actuators with wide-ranging applications.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/61/suppl/DC1 Materials and Methods Figs. S1 to S16 References (34, 35) Movies S1 to S8

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GAS GIANT PLANETS

In situ measurements of Saturn's ionosphere show that it is dynamic and interacts with the rings

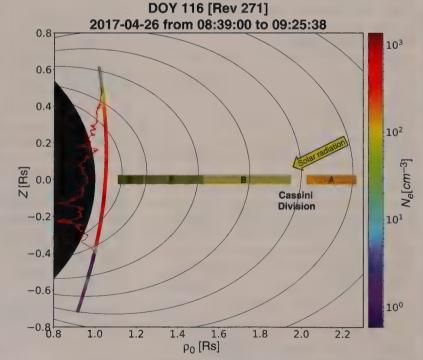
J.-E. Wahlund, 1* M. W. Morooka, L. Z. Hadid, A. M. Persoon, W. M. Farrell, 3 D. A. Gurnett, G. Hospodarsky, W. S. Kurth, S.-Y. Ye, D. J. Andrews, N. J. T. Edberg, A. I. Eriksson, E. Vigren

The ionized upper layer of Saturn's atmosphere, its ionosphere, provides a closure of currents mediated by the magnetic field to other electrically charged regions (for example, rings) and hosts ion-molecule chemistry. In 2017, the Cassini spacecraft passed inside the planet's rings, allowing in situ measurements of the ionosphere. The Radio and Plasma Wave Science instrument detected a cold, dense, and dynamic ionosphere at Saturn that interacts with the rings. Plasma densities reached up to 1000 cubic centimeters, and electron temperatures were below 1160 kelvin near closest approach. The density varied between orbits by up to two orders of magnitude. Saturn's A- and B-rings cast **a** shadow on the planet that reduced ionization in the upper atmosphere, causing a north-south asymmetry.

he gas giant planet Saturn is composed primarily of hydrogen and helium. The upper, tenuous parts of its atmosphere are partially ionized (producing ions and electrons), foremost by the solar extreme ultraviolet (EUV) radiation, but also by energetic particle impacts originating from the planet's magnetosphere and cosmic rays. The result is an electrically charged layer, the ionosphere, situated approximately between 300 and 5000 km altitude (1). By convention, the reference altitude of 0 km is at a pressure of 1 bar and is set to a distance of 60,268 km from the center of Saturn. So far, three methods of remote sensing have been used to infer the properties of Saturn's ionosphere: radio occultation (2-5), radio emission from Saturn's electrostatic discharges (6, 7), and millimeter-band observations of protonated molecular hydrogen (H₃⁺)(8). These measurement methods do not reveal smallscale variations (at scales below 1000 km); they only provide average constraints on the processes occurring in Saturn's ionosphere. In addition, the first two of these methods only derive the electron density, which, in the presence of abundant organic or water cluster ions (positively and negatively charged), does not give a representative

Fig. 1. Electron density (Ne, colored band) measured by the Cassini Langmuir probe on 26 April 2017.

The spacecraft crossed through the gap between the planet Saturn (solid black) and its rings (denoted D, C, B, Cassini division, and A), passing from north to south. The color code signifies the measured electron number density (N_e) , which is also shown as a red line (shown in linear scale: the density increases toward the left, with a maximum density of 1300 cm⁻³). Four dominant electron



density enhancements are mapped along Saturn's magnetic field to, or inside, the inner edge of the D-ring at the equator. The solar elevation angle was 26.7° during the event, and the shadows of the A-ring and part of the B-ring result in decreased ionization in the south. DOY, day of year; rev, orbit; R_S, Saturn radius; p_O, distance from Saturn.

picture of the true ionospheric densities (9, 10). Nevertheless, the radio occultation data set reveals a decrease in the plasma number densities toward lower latitudes and somewhat higher densities toward local dusk (2-5).

We present in situ measurements of Saturn's topside ionosphere from the Cassini spacecraft. The spacecraft first encountered the ionosphere around 09:00 UT on 26 April 2017 (Fig. 1) and continued to do so every ~6.5 days during the subsequent orbits. Data from the first 11 orbits are considered in this paper. The closest approach during the first encounter occurred at an altitude of 2800 km and varied from 2600 to 4000 km during the subsequent 10 crossings. Two types of data were collected using the Radio and Plasma Wave Science (RPWS) instrument package: Langmuir probe measurements and observations of whistler-mode emissions, from which the electron plasma frequency (f_{pe}) was determined (II). Above 1500 km altitude, the hydrogen ions (H⁺ and H₃⁺) are expected to be the dominant ionospheric species, whereas methane and other heavier species become important for the ionospheric chemistry below this altitude (12). All the crossings that we present occurred between 11 and 14 hours Saturn local time (LT) (table S1).

Figure 2 shows the altitude profiles of the electron and ion number densities (Fig. 2A) and the electron temperature (Fig. 2B) derived from the RPWS measurements during the first Cassini ring and ionosphere crossing on 26 April 2017. By comparison with Fig. 1, it can be seen that the altitude profiles in Fig. 2 really are convolutions of latitude and altitude. During this initial flyby of the ionosphere, the scale height varied between 1000 and 1500 km. The combination of a dense plasma (reaching 1000 cm⁻³ near closest approach) with a decreasing electron temperature (to below 0.1 eV or 1160 K) on approaching the planet indicates the detection of the cold and dense ionosphere of Saturn. Theoretical ionosphere modeling results indicate that electron temperatures near noon at 3000 km altitude could reach 500 K (12). However, the measured electron temperature data presented here are variable at altitudes up to 7000 km, contrary to the theoretical predictions.

From the inbound ion density measurements in Fig. 2A, we conclude that Saturn's ionosphere was dominated by H⁺ ions during this flyby. This conclusion holds in most of the data from all 11 flybys. One exception is the flyby on 4 June 2017 (orbit 277; Fig. 3), where RPWS detected the signature of a heavier dominant ion species [>18 atomic mass units (amu)] near the equator, which is also related to differences in ion and electron densities indicative of the presence of negatively charged nanometer-sized grains or cluster ions. Orbit 277 is also one of the crossings closest to the D-ring (13) and the

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most distant of the crossings, with closest approach near 4000 km altitude. We interpret this observation as a detection of ionized material from the nearby D-ring.

The rings cast shadows on Saturn. On the basis of data from the first 11 flybys, the A- and most of the B-ring must be opaque to solar EUV because very little plasma was detected in the regions within the geometric shadows cast by these rings (Fig. 2A and figs. S2 and S3). The dropout of ionization corresponding to the shadow from the middle of the B-ring near 1.7 Saturn radii (R_s) (Fig. 1) is at a location where there are SOI (Saturn orbit injection) observations of a B-ring source of whistler-mode emissions indicative of field-aligned currents (14) and a related plasma cavity (15). A plasma sink near the rings would lead to enhanced diffusion at magnetically connected higher latitudes (>35°), suppressing the plasma density at that point in the ionosphere. The Cassini division is somewhat less opaque to EUV, whereas no effects can be detected for the D- and C-rings (which thus must be EUV-transparent). The enhanced ionization of the ionosphere where it is illuminated by light passing through the Cassini division, compared with the area in the shadow of the opaque A- and B-rings (Fig. 2A), is detectable in all 11 flybys (figs. S2 and S3). The low levels of ionospheric plasma observed by RPWS in the shadowed regions (for latitudes between -20° and -40°) indicate that no major transport of plasma occurs from nearby regions. The lack of emitted photoelectrons detected by the Langmuir probe in this region confirms the low level of photoionization (figs. S2 and S3). Theoretical predictions of reduced ionization owing to the ring shadows may explain why radio emissions from Saturn's electrostatic discharges leak out from lower atmospheric layers past the ionosphere peak (16).

The observations put constraints on the possible transport of charged water products from the rings along conjugate magnetic field lines (also called ring rain), as indicated by remote infrared H_3^+ emissions (8, 17). A steady influx of water ions would react quickly with atmospheric hydrogen, recombining to neutral species, thereby removing the ionized component much faster than if only H⁺ recombined with electrons (18). An inflow of large quantities of heavy ions from the C-ring along connected magnetic flux tubes is not confirmed by the RPWS observations in the shadowed ionosphere region (Fig. 4). The question of ring rain at higher latitudes (>35°) is not yet constrained by the data.

Figure 4 shows the large variability and fine structure of Saturn's ionosphere. Maximum electron densities span a wide range, 50 to 1300 cm⁻³, which cannot be explained by a simple scale height model of a quiet ionosphere in photochemical equilibrium. One possibility is that a ring rainlike mechanism operates here, where variable amounts of water group (or molecular) ions originating from the D-ring material (near the equator or along connecting magnetic flux tubes) cause sporadic electron depletions. Electron depletions are evident within ±10° latitude during orbits 272, 273, 275, 277, 278, 280, and 281 (Fig. 4). These suppressed electron density dips vary between 50 and 200 cm⁻³. The equator crossings for orbits 276 and 277 occurred furthest away from the planet, around 4000 km altitude, which we expect to be inside the D-ring. An equatorial depletion of 50 cm⁻³ was detected during orbit 277, where the Langmuir probe also detected the presence of negatively charged cluster ions or nanometer-sized grains (Fig. 3). Orbit 276 does not show a clear depletion signature. However, it is difficult to explain the full dynamic range of almost two orders of magnitude between each flyby with this chemical process alone. There is no continuous evolution of plasma density from the first orbit to the last, and large variations can occur between adjacent orbits (e.g., orbits 277 and 278; Fig. 4).

The time constants for transport compete effectively with photochemistry, and thus the plasmas sampled depend strongly on dynamics. We therefore suggest that an electrodynamic interaction occurs between the ionosphere and the electrically charged D-ring via the strong magnetic field of Saturn. This would drive ionospheric ion outflows

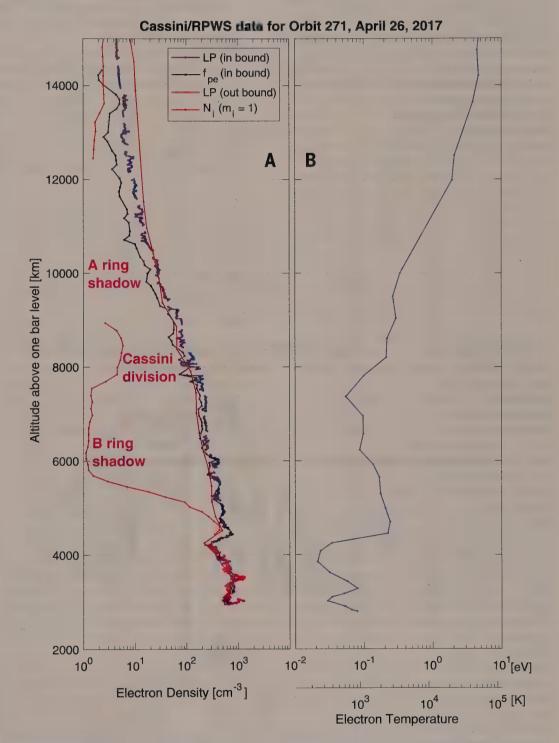


Fig. 2. Cassini RPWS altitude profiles of the ionosphere during the crossing on 24 April 2017. (A) Electron number density. (B) Inbound electron temperature. Two independent methods for estimating the inbound electron density (blue and black) gave almost identical results, confirming their validity. The Langmuir probe (LP) ion density $[N_i$, assuming the mass (m_i) of H^+ also produces values that agree with the estimated electron densities, confirming that hydrogen ions dominated during this flyby. The lower Langmuir probe electron densities over the outbound sector (magenta) indicate that much of the A- and part of the B-ring are opaque to ionizing extreme ultraviolet radiation. No whistler-mode cutoff data (black) exist for the outbound portion.

Fig. 3. RPWS data from an ionosphere crossing close to the D-ring of Saturn. (A) The Langmuir probe measured current (1) from voltage sweeps, (B) RPWS densities, and (C) inferred average ion mass. The electron densities (N_e) from three measurement methods (11) are lower than the estimated ion density (Ni) when Cassini passed the D-ring edge at 01:38 UT. The inferred average ion mass rose above 1 amu at this time. Ne, Fuh, electron density derived from the observed upper hybrid emission frequency.

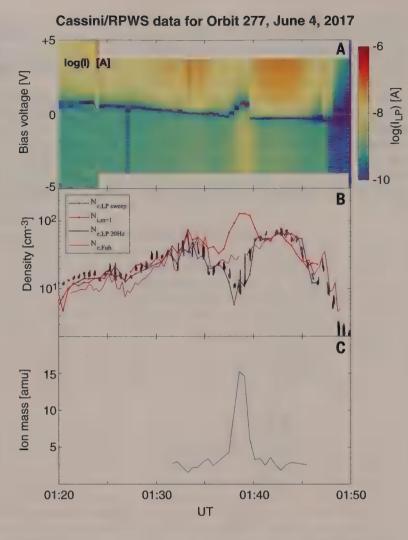
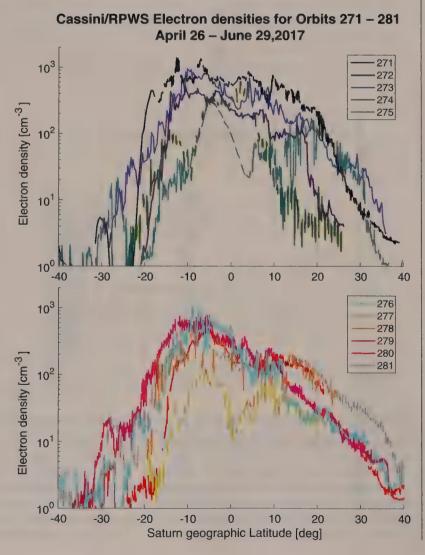


Fig. 4. RPWS electron number densities from 11 ionosphere crossings near the equatorial plane of Saturn. Densities for orbits 272 and 273 are based on whistler-mode cutoff data, whereas the rest are from the Langmuir probe measurements (the Langmuir probe was in the wake of the spacecraft during orbits 272 and 273). There are about 6.5 days between each flyby from 26 April to 29 June, all of which occurred close to 11 to 14 hours Saturn LT. The ionosphere exhibits large variability, with fine structure over these latitudinal-altitude cuts. The closest approach altitude occurs close to the geographic equator.



along magnetic flux tubes, leading to plasma structuring, as is observed along magnetic flux tubes above the aurora at Earth (19). The strong magnetic field of Saturn makes the rate of plasmaneutral collisions much smaller than the gyration frequency along the Cassini spacecraft trajectory; the ionospheric dynamo region, with coupling to neutral winds, is at a far lower altitude (20). The electrodynamics may instead be driven by the charged material in the D-ring (in Keplerian orbit) moving relative to the ionosphere (rotating with the planet), and a substantial crossmagnetic field current density may arise from the $\Delta \mathbf{V} \times \mathbf{B}$ term (where $\Delta \mathbf{V}$ is the relative speed of the two components and **B** is the magnetic field of Saturn). These ring currents then close via magnetic field-aligned currents to the conductive ionosphere below, with associated magnetic field-aligned ion flows along these flux tubes. Other possibilities for the variation in ionosphere dynamics involve strong longitudinal wind variations, coupled with variable EUV and X ionization levels, as is observed in Earth's ionosphere at ±20° latitude (21).

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/66/suppl/DC1 Materials and Methods Figs. S1 to S4 Table S1 Reference (22)

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STELLAR ASTROPHYSICS

An excess of massive stars in the local 30 Doradus starburst

F. R. N. Schneider, H. Sana, C. J. Evans, J. M. Bestenlehner, N. Castro, L. Fossati, G. Gräfener, N. Langer, O. H. Ramírez-Agudelo, C. Sabín-Sanjulián, S. Simón-Díaz, L. Tramper, P. A. Crowther, A. de Koter, S. E. de Mink, P. L. Dufton, M. Garcia, M. Gieles, O. Hénault-Brunet, A. Herrero, A. Herrero, L. Izzard, L. Dufton, V. Kalari, D. J. Lennon, L. Maíz Apellániz, M. Markova, F. Najarro, Ph. Podsiadlowski, J. Puls, W. D. Taylor, J. Th. van Loon, L. S. Vink, C. Norman^{26,27}

The 30 Doradus star-forming region in the Large Magellanic Cloud is \exists nearby analog of large star-formation events in the distant universe. We determined the recent formation history and the initial mass function (IMF) of massive stars in 30 Doradus on the basis of spectroscopic observations of 247 stars more massive than 15 solar masses (M_{\odot}). The main episode of massive star formation began about 8 million years (My) ago, and the star-formation rate seems to have declined in the last 1 My. The IMF is densely sampled up to 200 M_{\odot} and contains 32 \pm 12% more stars above 30 M_{\odot} than predicted by a standard Salpeter IMF. In the mass range of 15 to 200 M_{\odot} , the IMF power-law exponent is $1.90^{+0.37}_{-0.26}$, shallower than the Salpeter value of 2.35.

tarbursts are large star-formation events whose feedback affects the dynamical and chemical evolution of star-forming galaxies throughout cosmic history (1-3). They are found at low and high redshift, with the earliest starburst galaxies contributing to the reionization of the universe (2, 4). In such starbursts, massive stars $[\ge 10 \text{ solar masses } (M_{\odot})]$ dominate the feedback through intense ionizing radiation, stellar outflows, and supernova explosions. Because of large distances to most starbursts, analyses have so far been restricted to either photometric observations or composite spectra of entire stellar populations. In the former case, the high surface temperature of massive stars precludes the determination of accurate physical parameters because their colors are too similar (5), and, in the latter case, physical parameters of individual stars cannot be determined (6). Greater understanding can be obtained by spectroscopically examining individual stars within star-forming regions.

The stellar initial mass function (IMF) influences many areas of astrophysics because it determines the relative fraction of massive stars; that is, those that undergo supernova explosions and drive the evolution of star-forming galaxies. Much effort has therefore gone into understanding whether the IMF is universal or varies with local environmental properties (7, 8). Over the past few decades, evidence has accumulated that the IMF slope may be flatter than that of a Salpeter IMF (9)—in other words, there are more high-mass stars than expected—in regions of intense star formation (10–12). However, these studies are based on integrated properties of stellar populations, hampering the ability to infer IMFs.

The star-forming region 30 Doradus (30 Dor) lies within the Large Magellanic Cloud, a satellite galaxy of the Milky Way, and has a metallicity (total abundance of all elements heavier than helium) of ~40% of the solar value (13). At a distance of 50 kpc (14), 30 Dor is a nearby analog of distant starbursts and one of the brightest hydrogen-ionization (H $_{\rm II}$) regions in the local universe (15). With a diameter of ~200 pc, 30 Dor hosts several star clusters and associations and is similar in size to luminous H $_{\rm II}$ complexes in more distant galaxies (16).

Through the use of the Fibre Large Array Multi Element Spectrograph (FLAMES) (17) on the Very Large Telescope (VLT), the VLT-FLAMES Tarantula Survey (VFTS) (18) has obtained optical spectra of ~800 massive stars in 30 Dor, avoiding the core region of the dense star cluster R136 because of difficulties with crowding (18). Re-

peated observations at multiple epochs allow determination of the orbital motion of potentially binary objects. For a sample of 452 apparently single stars, robust stellar parameters—such as effective temperatures, luminosities, surface gravities, and projected rotational velocities—are determined by modeling the observed spectra (19). Composite spectra of visual multiple systems and spectroscopic binaries are not considered here because their parameters cannot be reliably inferred from the VFTS data.

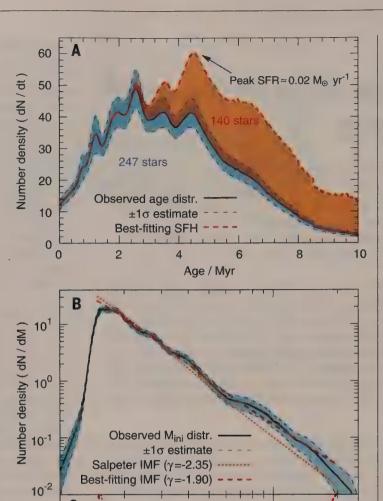
To match the derived atmospheric parameters of the apparently single VFTS stars to stellar evolutionary models, we use the Bayesian code Bonnsal, which has been successfully tested with high-precision observations of Galactic eclipsing binary stars (20). Bonnsai accounts for uncertainties in the atmospheric parameters and determines full posterior probability distributions of stellar properties, including the ages and initial masses of the VFTS stars (19). By summing these full posterior probability distributions of individual stars, we obtain the overall distributions of stellar ages and initial masses of massive stars currently present in 30 Dor (Fig. 1). These distributions are missing those stars that already ended their nuclear burning. However, given that we know both the present-day age and mass distributions, we can correct for these missing stars and derive the star-formation history (SFH) and IMF of massive stars in 30 Dor (19), allowing us to fully characterize this prototype starburst.

When determining the SFH and IMF, it is necessary to account for selection biases. The VFTS target selection implemented a magnitude cut, observing only stars brighter than the 17th magnitude in the V band (18). Compared with a full photometric census of massive stars in 30 Dor (21), the VFTS sample is ~73% complete. Although, owing to the magnitude limit. the VFTS is incomplete for stars $\lesssim 15 M_{\odot}$, the completeness shows no correlation with the V-band magnitude of stars more massive than $15\,M_{\odot}$ (19). Of the 452 stars with robust stellar parameters, 247 are more massive than 15 M_{\odot} and form the basis of our determination of the SFH and high-mass end of the IMF. Incompleteness corrections are applied to account for our selection process (19). We assume the high-mass IMF is a power-law function, $\xi(M) \propto M^{-\gamma}$, where M is the mass and γ the slope, and compute the SFH and corresponding prediction of the distribution of initial masses for different IMF slopes

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Fig. 1. Distributions of stellar ages and initial masses of massive stars in 30 Dor.

(A) Age and (B) initial mass (M_{ini}) distribution of the VFTS sample stars more massive than 15 Mo (black lines). Uncertainties are calculated by bootstrapping (19), and the 1_o regions are shaded blue. The best-fitting star-formation history (SFH) (A) and present-day distribution of initial masses (B) are plotted in red. For comparison, the expected present-day distribution of initial masses, assuming a Salpeter initial mass function (IMF), is also provided in (B) (note that these modeled mass distributions are no longer single power-law functions). About 140 stars above 15 M_☉ are inferred to have ended their nuclear burning during the last ≈10 My, and their contribution to the SFH is shown by the red shaded region in (A). The peak star-formation rate (SFR) extrapolated to the entire 30 Dor region is $\sim 0.02 M_{\odot} \text{ year}^{-1}$ (on the order of $\approx 1 M_{\odot} \text{ year}^{-1} \text{kpc}^{-2}$, depending on the exact size of 30 Dor). The age and mass distributions are number density functions (dN/dt and dN/dM) with respect to age t and mass M. γ is the power-law slope of the



IMF. (C) Ratio of modeled to observed present-day mass functions, illustrating that the Salpeter IMF model underpredicts the number of massive stars in our sample, particularly above 30 M_{\odot} .

1.4

1.2

0.8

0.6

0.4

10

Model / Obs

until we best match (i) the number of stars above a given mass and (ii) the observed initial-mass distribution (19).

We find that the observed distribution of initial masses of stars in 30 Dor is densely sampled up to ~200 M_{\odot} . It is shallower than that predicted by a Salpeter IMF with $\gamma = 2.35$, and the discrepancy increases with mass (Fig. 1C). Relative to the Salpeter index, we find an excess of $18.2^{+6.8}_{-7.0}$ ($32^{+\bar{1}2}_{-12}$ %) stars more massive than 30 M_\odot and $9.4^{+4.0}_{-4.6}$ (73^{+31}_{-36} %) stars more massive sive than $60 M_{\odot}$ (Fig. 2 and fig. S5; unless stated otherwise, uncertainties are 68.3% confidence intervals). The hypothesis that a Salpeter IMF can explain the large number of stars more massive than $30 M_{\odot}$ in our sample can thus be rejected with >99% confidence (19). The number of stars more massive than 30 M_{\odot} is best reproduced by an IMF slope of $\gamma = 1.84^{+0.18}_{-0.18}$ (Fig. 2). Using our second diagnostic, a leastsquares fit to the observed distribution of initial masses over the full mass range of 15 to $200 M_{\odot}$, our best fit is $\gamma = 1.90^{+0.37}_{-0.26}$ (Figs. 1 and 3), in agreement with our first estimate based on the number of massive stars $\geq 30 M_{\odot}$. Our high-mass IMF slope is shallower than the slope inferred by other studies for stars below $\approx 20 M_{\odot}$ in the vicinity of R136 (22, 23).

M_{ini} / M_o

100

The limitation of our sample to stars $\geq 15 M_{\odot}$ means that we can reconstruct the SFH of 30 Dor over the past ≈12 million years (My). When also considering the 1- to 2-My-old stars in R136 that were not observed within VFTS (24), we find that the star-formation rate in 30 Dor sharply increased ~8 My ago and seems to have dropped ~1 My ago (Fig. 1A). If the currently observed drop continues for another million years, the duration of the main star-forming event will be shorter than ~10 My. This result complements a recent study (23) that found a similar time-dependence of star formation around the central R136 star cluster in 30 Dor on the basis of photometric data of low- and intermediate-mass stars. We therefore conclude that star formation in the 30 Dor starburst is synchronized across a wide mass range.

Our results challenge the suggested 150 M_{\odot} limit (25) for the maximum birth mass of stars. The most massive star in our sample, VFTS 1025 (also known as R136c), has an initial mass of $203^{+40}_{-44} M_{\odot}$ (19). From stochastic sampling experiments (19), we exclude maximum stellar birth masses of more than $500\,M_\odot$ in 30 Dor with 90% confidence because we would otherwise expect to find at least one star above 250 M_{\odot} in our sample. Our observations are thus consistent with the claim that stars with initial masses of up to 300 M_{\odot} exist in the core of R136 (26).

Approximately 15 to 40% of our sample stars are expected to be products of mass transfer in binary star systems (27). Binary mass transfer in a stellar population produces a net surplus of massive stars and rejuvenates stars such that they appear younger than they really are (28). Mass accretion alone biases the inferred IMF slope to flatter values, whereas rejuvenation steepens it. Taken together, we calculate that these two effects roughly cancel out in our case and, thus, binary mass transfer cannot explain the difference between our inferred IMF and that of Salpeter (19). Also, our final sample of stars contains unrecognized binaries, but they do not affect our conclusions (19).

The core of the R136 star cluster is excluded from the VFTS, but stars ejected from R136 (socalled runaway stars) may enter our sample. Runaway stars are biased toward high masses (29) and thus flatten the upper IMF. However, star clusters such as R136 typically eject about 5 to 10 stars above 15 M_{\odot} (30, 31), which is insufficient to explain the expected excess of 25 to 50 stars above 30 M_{\odot} in 30 Dor, after correcting for the completeness of our sample and that of the VFTS (19).

We conclude that the 30 Dor starburst has produced stars up to very high masses ($\gtrsim 200 M_{\odot}$), with a statistically significant excess of stars above $30 M_{\odot}$ and an IMF shallower above $15 M_{\odot}$ than a Salpeter IMF. Measuring the IMF slope above 30 to $60 M_{\odot}$ has proven difficult (7), and, in general, large uncertainties in the high-mass IMF slope remain (32). This raises the question of whether star formation in 30 Dor proceeded differently. It has been suggested that starburst regions themselves provide conditions for forming relatively more massive stars by the heating of natal clouds from nearby and previous generations of stars (33). Alternatively, a lower metallicity may lead to the formation of more massive stars because of weaker gas cooling during star formation. An IMF slope shallower than the Salpeter value may then be expected at high redshift when the universe was hotter and the metallicity lower (33, 34).

Because massive-star feedback increases steeply with stellar mass, it is strongly affected by the IMF slope. Comparing an IMF slope of $\gamma = 1.90^{+0.37}_{-0.26}$ to the Salpeter slope, we expect 70⁺¹⁰₋₆₀% more core-collapse supernovae and an increase of supernova metal-yields and hydrogen ionizing radiation by factors of $3.0^{+1.6}_{-1.8}$ and $3.7^{+2.4}_{-2.4}$, respectively (19). The formation rate of black holes increases by a factor of $2.8^{+1.0}_{-1.6}$ (19), directly affecting the expected rate of black hole mergers detected by their gravitational wave signals. We also expect an increase in the predicted number of exotic transients that are preferentially found in starbursting, metalpoor dwarf galaxies such as long-duration gammaray bursts (35) and hydrogen-poor superluminous supernovae (36). Many population synthesis models and large-scale cosmological simulations Expected number of massive stars in our sample initially more massive than (A) $30 M_{\odot}$ and (B) $60 M_{\odot}$ as a function of the IMF slope y (black solid lines). The blue and red shaded areas indicate the 68 and 95% confidence intervals (CIs). respectively, of the observed number of stars (see fig. S5). The IMF slopes best reproducing the observed number of stars and the associated 68% intervals are indicated by the vertical dashed lines and gray shaded regions and correspond to $\gamma = 1.84^{+0.18}_{-0.18}$ and $\gamma = 1.84^{+0.22}_{-0.17}$ for stars more massive than 30 Mo and 60 M_{\odot} , respectively.

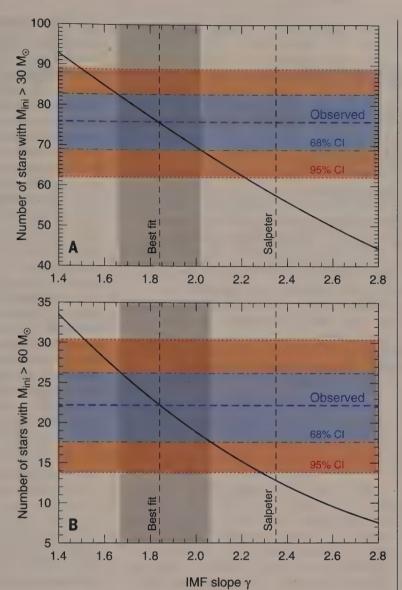
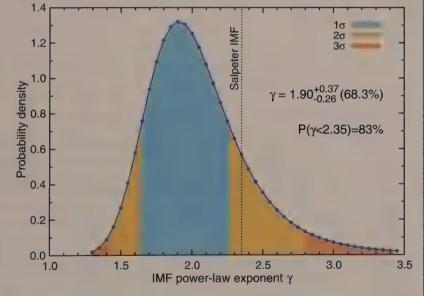


Fig. 3. Probability density function of the inferred IMF slope in 30 Dor. Results are based on a χ² powerlaw fitting over the mass range of 15 to 200 M_{\odot} . The shaded areas represent 1_o, 2_o, and 3_o confidence regions, and the slope of the Salpeter IMF is indicated by the vertical dashed line. Our inferred IMF is shallower than that of Salpeter ($\gamma = 2.35$), with 83% confidence.



assume an IMF that is truncated at $100 M_{\odot}$. Compared with such models, the various factors estimated above are even larger (19).

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/69/suppl/DC1 Materials and Methods Supplementary Text Figs. S1 to S10 Tables S1 to S3 References (37-116) Data S1 to S3

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POLYMERS

Mechanically robust, readily repairable polymers via tailored noncovalent cross-linking

Yu Yanagisawa, 1,2 Yiling Nan, 1 Kou Okuro, 1 Takuzo Aida 1,2 ×

Expanding the range of healable materials is an important challenge for sustainable societies. Noncrystalline, high-molecular-weight polymers generally form mechanically robust materials, which, however, are difficult to repair once they are fractured. This is because their polymer chains are heavily entangled and diffuse too sluggishly to unite fractured surfaces within reasonable time scales. Here we report that low-molecularweight polymers, when cross-linked by dense hydrogen bonds, yield mechanically robust yet readily repairable materials, despite their extremely slow diffusion dynamics. A key was to use thiourea, which anomalously forms a zigzag hydrogen-bonded array that does not induce unfavorable crystallization. Another key was to incorporate a structural element for activating the exchange of hydrogen-bonded pairs, which enables the fractured portions to rejoin readily upon compression.

ealable polymers with enhanced longevity and reliability are particularly attractive as next-generation materials for the realization of a sustainable society (1-3). On the basis of their healing mechanisms, such materials are classified as exhibiting extrinsic (1, 2) or intrinsic (1, 3) healing behavior. Extrinsic healing depends on the presence of finely dispersed small capsules or vascular networks that separately entrap monomers and initiators that are mixed when the containers are broken, resulting in the formation of new polymer chains that connect the fractured parts (4, 5). This approach is reliable, but it allows the material to heal only a limited number of times. In contrast, intrinsic mechanisms enable polymeric materials to heal many times, in some cases at ambient temperatures, through the reorganization of dynamic covalent bonds (6-10) or through noncovalent interactions, mostly involving hydrogen bonds (H bonds) (11-15). These healable materials are usually soft and deform-

TUC₁₂

UEG₃

8,700

6,600

36

13,900

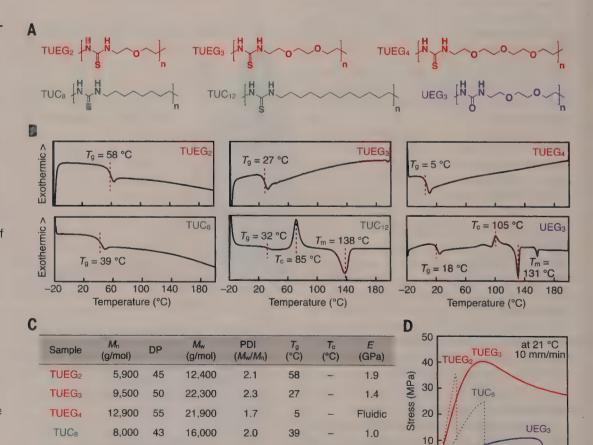
able. Some healable materials with high mechanical robustness have also been developed by cross-linking with dynamic covalent bonds. However, in most cases, heating to high temperatures (on the order of 120°C or more) to reorganize their cross-linked networks is necessary for the fractured portions to repair (6-8).

In general, noncrystalline, high-molecularweight polymers form mechanically robust materials owing to the entanglement of long polymer chains. However, once the materials are fractured, they are difficult to repair unless they are heated to melt, because the entangled polymer chains diffuse too sluggishly to unite fractured portions within reasonable time scales (2). On the other hand, low-molecular-weight polymers, when they are noncovalently cross-linked by H bonds, may be elaborated into mechanically robust vet healable polymeric materials, because their dynamic properties are modulable by changing the cross-linking density. So far, rubber-like soft materials (11) and thermoplastic elastomers (14) with H-bonding motifs have been designed to heal upon gentle compression. However, the presence of a large number of H bonds often leads to crystallization or clustering of polymeric materials, thereby making them brittle (16-18). In other words, high mechanical robustness and healing ability tend to be mutually exclusive.

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Fig. 1. Molecular structures and characterization of poly(ether-thiourea) TUEG3 and its reference polymers. (A) Schematic structures of poly(ether-thioureas) with diethylene glycol (TUEG₂), triethylene glycol (TUEG₃), and tetraethylene glycol (TUEG₄) as spacers; schematic structures of poly(alkylene-thioureas) with octamethylene (TUC₈) and dodecamethylene (TUC₁₂) chains as spacers; and schematic structure of a poly(ether-urea) with triethylene glycol (UEG₃) as a spacer. (B) Differential scanning calorimetry thermograms for TUEG₂, TUEG₃, TUEG₄, TUC₈, TUC₁₂, and UEG₃ on the second heating from -20°C to 200°C at a rate of 10° C/min. $T_{\rm g}$, $T_{\rm c}$, and $T_{\rm m}$ denote the glasstransition temperature, crystallization temperature, and melting temperature, respectively. (C) Characterization of polymers. M_n is the number-average molecular weight, DP is the degree of polymerization as estimated by ¹H NMR end-group analysis, M_w is the weightaverage molecular weight obtained by multiangle light scattering, PDI is the polydispersity index (M_w/M_p) , and E is the elastic modulus evaluated from the stress-strain curve in tensile testing (D). (D) Stress-strain curves of TUEG₂, TUEG₃, TUC₈, TUC₁₂, and UEG₃ at 21°C.



32

18

2.1

85

02

0.2

10 Strain (%)

Here we report that poly(ether-thioureas) such as TUEG2 and TUEG3 (Fig. 1A) anomalously form amorphous materials, despite carrying dense Hbonding thiourea units. These materials are highly mechanically robust yet can be readily repaired by compression at fractured surfaces. Why are they amorphous despite carrying a large number of H-bonded thiourea units? We found that their H-bonded thiourea arrays are geometrically nonlinear (less ordered), so that they do not induce crystallization. Originally, we prepared TUEG₃ as a synthetic intermediate for guanidinium ion-appended polymers that strongly bind to oxyanionic groups in biomacromolecules (19-21). During the isolation of TUEG₃, we noticed that cut surfaces of this polymer are self-adhesive, despite their rigid and nontacky nature. Cut pieces of a 2-mm-thick rectangular sheet of TUEG₃ (10 mm by 20 mm) formed a merged sheet that withstood a 300-g load when their fractured surfaces were manually compressed for 30 s at an ambient temperature of 21°C (fig. S1).

TUEG₃ (Fig. 1A) can be prepared by a onepot polycondensation of commercially available monomers (supplementary materials, p. S4). It was characterized by means of ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy and elemental analysis. ¹H NMR end-group analysis and multiangle light-scattering studies showed that its number- and weight-average molecular weights (M_n and M_w) were 9500 and 22,300, respectively (Fig. 1C). TUEG3 is amorphous, as shown by its differential scanning calorimetry profile on the second heating (Fig. 1B). No sharp peaks owing to crystallization appeared in the temperature range from -20° to 200°C at any scan rate from 1° to 10°C/min, whereas a broad peak corresponding to a glass transition (T_g) appeared at 27°C (fig. S8B). Furthermore, in its x-ray diffraction profile recorded at 22°C, TUEG₃ exhibited a broad diffraction peak centered at $2\theta = 23^{\circ}$, typical of an amorphous material (fig. S10B). The thiourea group is special as an H-bonding motif (22), because UEG₃, the urea analog of TUEG3, is semicrystalline (Fig. 1, A and B, and fig. S10E).

According to a thorough crystallographic study of the geometries of H-bonded thiourea and urea derivatives (23), the former mostly form nonlinear zigzag arrays of H-bonded thiourea units adopting cis/trans and strained trans/trans conformations (I in Fig. 2A), whereas the latter form linear arrays of H-bonded urea units adopting only a trans/trans conformation (II in Fig. 2A). We consider that the amorphous nature of TUEG₃ originates from such less ordered, nonlinear geometries of its H-bonded thiourea units. Fourier transform infrared (FTIR) spectroscopy of TUEG₃ at 22°C showed broad vibrational bands at around 3290 and 3060 cm⁻¹ (Fig. 2B), the former of which can be assigned to the NH stretching vibration, whereas the latter is characteristic of an NH deformation vibration of nonlinearly H-bonded thiourea units (24). Because the estimated energy gap between the cis/trans and trans/trans conformations is 2.9 to 4.2 kJ/mol (25), these two conformers likely coexist in the polymer matrix of TUEG₃. Semicrystalline UEG₃, the urea analog of TUEG₃, did not display an IR absorption at around 3060 cm⁻¹ (Fig. 2B). This is in accord with a previous report that H-bonded urea units adopt only a trans/trans conformation (Fig. 2, A and B) (23). The contrasting FTIR spectral features observed for amorphous TUEG3 and semicrystalline UEG3 highlight an important principle-namely, that densely located thiourea units, like urea units, tightly cross-link the polymer main chains through H-bonding interactions but do not induce crystallization because the resultant H-bonded arrays are nonlinear and less ordered.

To follow up on our preliminary healing test of TUEG₃ (fig. S1), we fabricated tensile bars by a method analogous to that used for the preparation of the polymer sheet, then subjected the bars to tensile tests (test rate, 10 mm/min) at an ambient temperature of 21°C. The stressstrain curve (red solid curve, Fig. 1D) displayed an abrupt increase in stress of as much as 45 \pm 8 MPa, and the sample yielded at an applied strain of $6 \pm 2\%$. After this yielding stage, the specimen elongated by up to 393 ± 5% before fracture (fig. S11). The elastic modulus of TUEG₃, as estimated from the initial slope of the stressstrain curve (Fig. 1D), was 1.4 GPa. Because dynamic mechanical properties are important for understanding the healing events at fractured portions, we performed temperature-dispersion tests for TUEG3 at 200° to 0°C (5°C/min) at a constant frequency (ω) of 10 rad/s. The storage

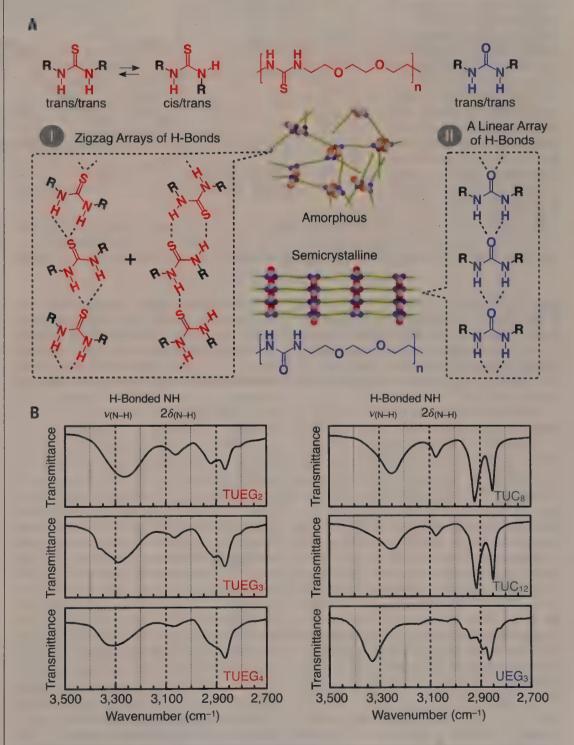
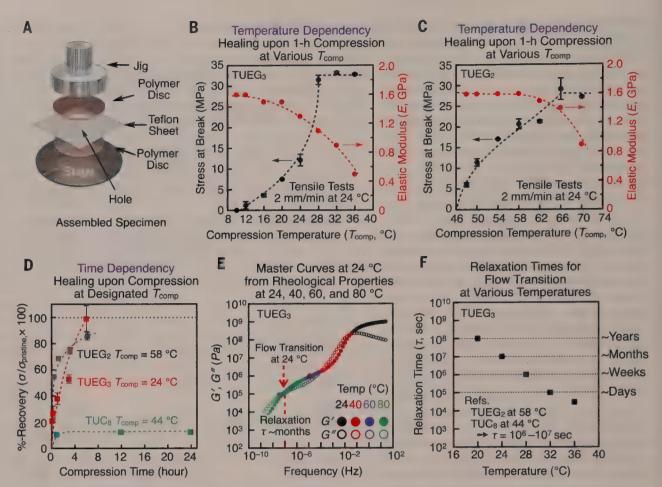


Fig. 2. Hydrogen-bonding interactions of thiourea and urea moieties in polymer matrices. (A) Schematic representations of the H-bonding modes of thiourea and urea. (B) FTIR spectra (bulk) at 22°C of poly(ether-thioureas) TUEG2, TUEG3, and TUEG4; poly(alkylene-thioureas) TUC8 and TUC₁₂; and poly(ether-urea) UEG₃.

Fig. 3. Healing behaviors of polymeric thiourea derivatives upon compression. Healing tests using a rheometer (26) are illustrated in (A) to (D). (A) Schematic representation of the assembled specimen. (B and C) Elastic moduli (E, red circles) of poly(ether-thioureas) (B) TUEG₃ and (C) TUEG2 at various temperatures, as estimated by compression testing (10 mm/min), and stresses at break for the assembled specimens (black circles) prepared by compression for 1 hour at various temperatures under a constant applied stress of 1.0 MPa. (D) Percentage recoveries of the stress at break for assembled specimens of TUEG2, TUEG3, and TUC₈ ($\sigma_{\text{pristine}} = 26.5 \pm 0.3$) 31.7 ± 0.5, and 39.1 ± 1.6 MPa, respectively) prepared by compression for various periods of time at $T_{\text{comp}} = 58^{\circ}$, 24°, and 44°C, respectively, under a constant applied stress of 1.0 MPa. (E) Master curves for TUEG3

at a reference temperature of



24°C, prepared by the time-temperature superposition treatment of storage modulus (G') and loss modulus (G") values obtained at 24°, 40°, 60°, and 80°C in frequency-dispersion tests [frequency (ω) = 628 to (6.28 × 10⁻³) rad/s] at an applied strain of 0.05 or 0.1%. (**F**) Relaxation times (τ) of TUEG₃ for the flow transition, estimated from the crossover points of G' and G" at various temperatures (20° to 36°C) (fig. S21).

modulus (G') and loss modulus (G") curves of TUEG₃ allowed for confirmation of the glass transition at around 30°C (fig. S12B). TUEG3 exhibited a rubber plateau region, typical of long polymer chains upon being entangled, at around 50° to 70°C, despite the fact that its molecular mass is less than 10 kDa.

We then tested the healing of TUEG₃ by a method analogous to that reported by Corté and co-workers (26). A 0.1-mm-thick sheet of Teflon (10 mm by 10 mm) with a 1-mm-diameter hole at its center (Fig. 3A) was sandwiched between two discs of TUEG3 (diameter, 8 mm; thickness, 1.0 mm). This assembled specimen was set on a rheometer stage, gently pressed with a jig, then annealed for 20 min at 90°C (hot melt), so that the touching parts of the upper and lower discs merged inside the hole area of the Teflon sheet. The resultant specimen was cooled to 24°C at a rate of 5°C/min, then the stage and jig in the rheometer were rapidly separated from one another at a rate of 100 mm/min to cause brittle fracture in the merged part of the specimen. After 3 min, the fractured surfaces were mechanically brought back into contact and compressed at the designated temperature under a constant applied stress. The compressed specimen was then subjected to tensile testing (tensile rate, 2 mm/min) to determine the stress at fracture.

Although the contact area between the upper and lower discs was only 0.79 mm², the tensile tests were highly reproducible (fig. S13). Even when the contact area was enlarged from 0.79 to 1.76, 3.14, or 19.6 mm² (figs. S14 and S15), the results obtained were consistent (fig. S16). When the applied compression stress was increased from 0.12 to 1.0 MPa, the recovery leveled off at 0.4 MPa after the initial abrupt increase (fig. S17). On the basis of these results, we carried out systematic healing tests by setting the contact area and applying compression stress at 0.79 mm² and 1.0 MPa, respectively. Figure 3B shows the stresses at break (black circles) observed for the assembled specimens of TUEG3 prepared by compression for 1 hour at various temperatures $(T_{\text{comp}} = 10^{\circ} \text{ to } 36^{\circ}\text{C})$. The elastic moduli (red circles) of the compressed specimens at ≤32°C were high (≥0.9 GPa). Figure 3D shows the percentage recoveries of the stresses at break ($\sigma/\sigma_{\text{pristine}} \times 100$) for TUEG3; the specimen compressed at 24°C completely recovered its original mechanical strength ($\sigma_{pristine}$) in 6 hours. Accordingly, the interfacial adhesion energy was estimated to be $\sim 0.9 \text{ J/m}^2$ (supplementary materials, p. S4). When T_{comp} was raised from 24°C to the higher range of 28° to 36°C, the specimen completely recovered within 1 hour (Fig. 3B), and the healing could be repeated multiple times without noticeable deterioration (fig. S18). Even at T_{comp} = 12°C, the specimen recovered partially.

On the basis of the frequency-dispersion properties of the G' and G" values at 20° to 100°C with $\omega = 628$ to (6.28×10^{-3}) rad/s (figs. S19 and S20), we estimated the relaxation times (τ) for

the flow transition of TUEG₃ by using their master curves (27) at 24° to 32°C (Fig. 3E and fig. S21). The τ values were in the range of 10⁷ s (on the order of months) to 10⁵ s (on the order of days) (Fig. 3F), far longer than the compression times required for TUEG₃ to heal completely at T_{comp} = 24° to 32°C (≤6 hours). This contrast suggests that the rapid healing behavior of TUEG₃ is not a consequence of the flow of polymer chains (11, 14) but is dominated by a much more rapid process.

Other polymeric thiourea derivatives including TUEG2, TUEG4, TUC8, and TUC12 (Fig. 1A)in which diethylene glycol, tetraethylene glycol, octamethylene, and dodecamethylene spacer units, respectively, connect the thiourea unitswere synthesized. Like TUEG3, TUEG2 and TUEG4 were both amorphous and exhibited only single, broad glass-transition peaks at 58° and 5°C, respectively (Fig. 1B and figs. S8 and S10). TUC₈ was also amorphous ($T_g = 39$ °C), whereas TUC₁₂ was semicrystalline (Fig. 1B and figs. S8 and S10). As in the case of TUEG₃, the FTIR spectra (Fig. 2B) of these reference polymers exhibited NH stretching and deformation vibrations between 3250 and 3300 cm⁻¹ and at around 3,060 cm⁻¹, respectively, the latter of which is typical of nonlinear H-bonded arrays of thiourea units (23).

We investigated the tensile behaviors of these polymers and of UEG3 at an ambient temperature of 21°C with a tensile rate of 10 mm/min (Fig. 1D). In contrast to TUEG₃, all of these polymers were highly brittle, except TUEG4, which was

too fluid-like to be tested. However, just below their glass-transition temperatures, TUEG2 did not fracture in a brittle manner but yielded like TUEG3, whereas TUC8 again showed brittle fracture (fig. S23). We then conducted healing tests on amorphous $TUEG_2$ and TUC_8 at $T_{comp} = 58^{\circ}$ and 44°C, respectively. These temperatures were used to compare the healing behaviors of these polymers with those of TUEG3 at 24°C (Fig. 3D), because their viscosities, as determined by shear creep tests, were comparable to one another $(2.5 \times 10^9 \text{ Pa·s for TUEG}_2 \text{ at } 58^{\circ}\text{C}, 2.2 \times 10^{-9} \text{ Pa·s for TUEG}_2)$ 10^9 Pa·s for TUC₈ at 44°C, and 3.3×10^9 Pa·s for TUEG₃ at 24°C) (fig. S25). Figure 3D shows that TUEG₂ at $T_{\text{comp}} = 58^{\circ}\text{C}$ exhibited an 85% recovery of its mechanical strength upon compression for 6 hours. Even when T_{comp} was lowered to 48°C (Fig. 3C, black circles), compressed TUEG₂ showed a partial recovery (22%) in 1 hour, although the elastic modulus of TUEG₂ at temperatures up to 70°C remained high (≥1 GPa) (Fig. 3C, red circles). In contrast, TUC, barely healed, even upon compression at 44°C over a long period of 24 hours.

Analogously to the case of TUEG3, we evaluated the relaxation times of TUEG2 and TUC8 for the flow transition (fig. S22) from their temperaturedispersion (fig. S12) and frequency-dispersion properties (figs. S19 and S20). As shown in fig. S22. TUEG₂ and TUC₈ exhibited a rubber plateau region similar to that of TUEG₃. Furthermore, both of these polymers showed extremely slow relaxation properties at T_{comp} [$\tau = 10^7 \text{ s}$ (on the order of months) to 10⁶ s (on the order of weeks)].

Why can TUEG2 as well as TUEG3 readily heal despite such slow diffusion dynamics? We consider that their healing properties are dominated by a segmental motion such as the exchange of H-bonded thiourea pairs, leading to the interpenetration of polymer chains at the fractured portions upon compression (28). The H-bond exchange of thiourea pairs should occur far more rapidly than the flow relaxation of these polymers (Fig. 3E and figs. S21 and S22). Consequently, virtually frozen TUEG2 and TUEG3 can heal only in a few hours. Then why does TUC₈ hardly heal? This question prompted us to consider a special role of the spacer units that connect the thiourea units. Arrhenius plots of the intersection frequencies for the G' and G''curves (fig. S20) allowed us to estimate the apparent activation energies (E_a) for the slippage of polymer chains (Fig. 4A). As shown in Fig. 4B, the E_a values for TUEG₂, TUEG₃, and TUC₈ were 171, 149, and 203 kJ/mol, respectively. In H-bonded polymeric materials, polymer chains presumably slip and interpenetrate by exchanging their H-bonded pairs. According to the Flory-Huggins interaction parameters (χ) of the model structures in Fig. 4C, thiourea is much more miscible with ethers than with hydrocarbons. In ethereal media, the ether oxygen atoms might facilitate the exchange of H-bonded thiourea pairs by serving as temporal H-bond acceptors (Fig. 4D); in other words, the ether-containing TUEG2 and TUEG₃ use this mechanism to lower the energy barrier for the slippage of their polymer chains.

This is not the case in TUC₈, because the thiourea group is incompatible with hydrocarbons (Fig. 4C). It is therefore reasonable that the E_a value for TUC₈ is larger than those for ethercontaining TUEG2 and TUEG3, and the same might hold true even in a lower temperature range.

This work provides the essential structural elements for the design of mechanically robust

yet healable polymeric materials: (i) relatively short polymer chains that permit greater segmental motions, (ii) tight cross-links by a large number of H bonds for better mechanical properties, (iii) nonlinear (less ordered) H-bond arrays that do not induce crystallization, and (iv) implemented mechanisms to facilitate the exchange of H-bonded pairs. The triethylene glycol spacer

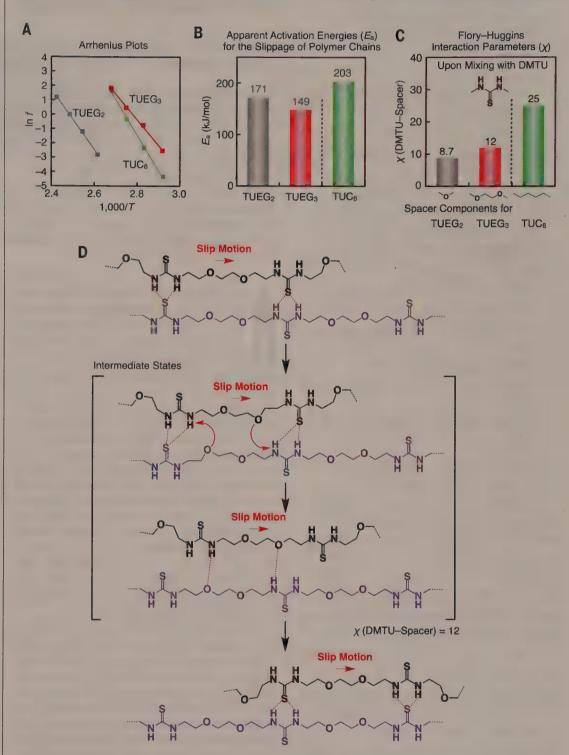


Fig. 4. Slip motions of polymer chains in poly(ether-thiourea) and poly(alkylene-thiourea) **through exchange of H-bonded pairs.** (A) Arrhenius plots of the intersection frequencies $(f = \omega/2\pi)$ for the G' and G" curves of poly(ether-thioureas) TUEG2 and TUEG3 and poly(alkylene-thiourea) TUC8. estimated from frequency sweep tests $[\omega = 100 \text{ to } 0.05 \text{ rad/s}]$ at a constant applied strain of 0.1% in the temperature range for the flow transition (fig. S20). (B) Apparent activation energies (Ea) of TUEG2, TUEG₃, and TUC₈ for the slippage of polymer chains, estimated from the Arrhenius plots (C) Flory-Huggins interaction parameters χ (DMTU-spacer) for N,N'-dimethylthiourea (DMTU) in combination with dimethyl ether (a model mixture for TUEG2), ethylene glycol dimethyl ether (a model mixture for TUEG₃), or hexane (a model mixture for TUC₈). These values were estimated from the corresponding Hildebrand solubility parameters, determined by the Fedors method (29, 30). (D) Proposed mechanism of how the exchange of H-bonded thiourea pairs in TUEG₃ is enhanced.

that connects the thiourea units in TUEG₃ optimally modulates the activation energy for the exchange of H-bonded thiourea pairs; consequently, the polymeric material, although highly cross-linked noncovalently, can heal by compression without heating.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/72/suppl/DC1 Materials and Methods Figs. S1 to S25

Reference (31)
Movie S1

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TOPOLOGICAL MATTER

Observation of the quantum spin Hall effect up to 100 kelvin in a monolayer crystal

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A variety of monolayer crystals have been proposed to be two-dimensional topological insulators exhibiting the quantum spin Hall effect (QSHE), possibly even at high temperatures. Here we report the observation of the QSHE in monolayer tungsten ditelluride (WTe₂) at temperatures up to 100 kelvin. In the short-edge limit, the monolayer exhibits the hallmark transport conductance, \sim e²/h per edge, where e is the electron charge and h is Planck's constant. Moreover, magnetic field suppresses the conductance, and the observed Zeeman-type gap indicates the existence of Kramers degenerate point and the importance of time-reversal symmetry for protection from elastic backscattering. Our results establish the QSHE at temperatures much higher than in semiconductor heterostructures and allow for exploring topological phases in atomically thin crystals.

time-reversal (TR) invariant topological insulator (TI) in two dimensions, also known as a quantum spin Hall (QSH) insulator, can be identified by its helical edge modes (1-4). So far, evidence for the helical edge mode in two-dimensional (2D) TIs, particularly quantized transport, has been limited to very low temperatures (i.e., near liquid helium temperature) in HgTe and InAs/GaSb quantum wells (5, 6). In the search for high-temperature TIs, substantial efforts have focused on a variety of atomically thin materials (7-14), which hold the promise of advancing the field of topological physics using the tools developed for 2D crystals. However, experimental observation of the quantum spin Hall effect (QSHE) in monolayer systems is challenging, often owing to structural or chemical instabilities (9, 15-17). Indications of a hightemperature QSH phase in bulk-attached bismuth bilayers have been reported (7, 18, 19), but a conclusive demonstration is still lacking.

Among the proposals for atomically thin TIs are monolayer transition metal dichalcogenides (TMDs), materials that are either 2D semiconductors or semimetals depending on their structural phase (9). Calculations suggest that an inverted band gap can develop in 1T' TMD monolayers, resulting in a nontrivial Z_2 topological phase (9, 20). Recent experiments have shown promising results (12–14), including that monolayer WTe₂ exhibits a ground state with an insulating interior and conducting edges associated with a

zero-bias anomaly (12), distinct from its multilayer counterparts (12, 21). Here we observe the QSHE in WTe₂ monolayers and identify this 2D material as an atomically layered TI with conductance $\sim e^2/h$ per edge at high temperatures, where e is the electron charge and h is Planck's constant.

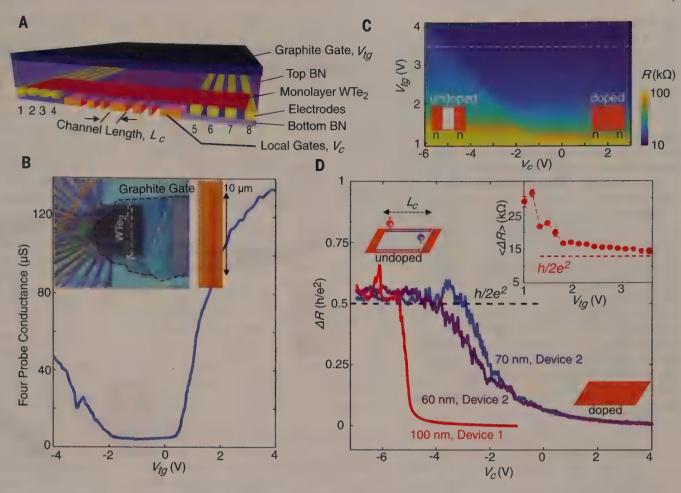
QSH transport through a 2D TR-invariant TI should exhibit the following characteristics: (i) helical edge modes, characterized by an edge conductance that is approximately the quantum value of e^2/h per edge (5); (ii) saturation to the conductance quantum in the short-edge limit (22); and (iii) suppression of conductance quantization upon application of a magnetic field, owing to the loss of protection by TR symmetry (5, 23, 24). Signatures of a Zeeman gap should be seen if the Kramers degeneracy (Dirac point) is located inside the bulk band gap. To date, simultaneous observation of the above criteria in existing 2D TI systems is still lacking (5, 6, 22, 23, 25), prompting the search for new QSH materials.

To check the above criteria in monolayer WTe₂, we fabricated devices with the structure depicted in Fig. 1A [see (26) and figs. S1 and S2]. The goal of the design was threefold: to ensure an atomically flat, chemically protected channel (no flake bending or exposure) by fully encapsulating the flake with hexagonal boron nitride (15, 21); to minimize the effect of contact resistance; and to enable a length-dependence study on a single device. Our devices generally contain eight contact electrodes, a top graphite gate, and a series of in-channel local bottom gates with length $L_{\rm c}$ varying from 50 to 900 nm. The monolayer flakes are carefully selected to have a long strip shape, typically a few um wide and about 10 μm long (table S1). Figure 1B shows a typical measurement of the four-probe conductance (in device 1) across all the local gates (~8 µm long) as a function of top-gate voltage, $V_{\rm tg}$. A finite

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Fig. 1. Device structure and resistance neer h/2e2. (A) Schematic of the device structure. BN. boron nitride. (B) Fourprobe conductance measurement at 4 K of device 1 as a function of V_{tg} across all the local gates, which are floating. Inset shows the optical image of device 1 (left) and the corresponding monolayer WTe2 flake before fabrication (right). (C) Color map of the flake resistance tuned by $V_{\rm tg}$ and the 100-nm-wide local gate V_c at 4 K. Two regions are separated by a step in the resistance. which distinguishes the doped and undoped local channels, as depicted by the inset schematics. Ω , ohm. (**D**) ΔR versus V_c for the 100-nm-wide gate on device 1 at $V_{\rm tg} = 3.5 \text{ V}$ [white dashed line in (C)] and the 60- and 70-nm-



wide gates on device 2 at V_{tg} = 4.1V (taken at 5 K). For clarity, the two curves from device 2 are offset by +3 V along the x axis. Inset shows the average step height $<\Delta R>$, extracted from (C), as a function of $V_{\rm tg}$, showing a clear saturation toward $h/2e^2$ for large $V_{\rm tg}$.

conductance plateau develops around V_{tg} = 0 V. This characteristic feature for monolayer WTe2 stems from conduction along the edges (12). The measured value is highly sensitive to contact properties (12), which prevents observation of the intrinsic edge conductance. We overcome this obstacle in our devices through selective doping of the flake using a combination of global top and local bottom gates. A short transport channel with length $L_{\rm c}$ can be selectively defined by a local gate voltage V_c , whereas the rest of the flake is highly doped by V_{tr} to secure good contact to the electrodes (see fig. S3 for dI/dV characteristics, where $dI \sim$ 1 nA is the applied ac current). Figure 1C maps out the resistance R in the same device as a function of $V_{\rm tg}$ and $V_{\rm c}$ (for a local gate with $L_{\rm c}$ = 100 nm). The step structure indicates a transition from a bulk-metallic state (doped) to a bulkinsulating state (undoped) within the locally gated region. We define the offset resistance, $\Delta R = R(V_c) - R(V_c = -1V)$, as the resistance change from the value in the highly doped limit ($V_c = -1V$, in this case). Figure 1D shows a ΔR trace (red curve) extracted from Fig. 1C (dashed white line in Fig. 1C), where $V_{\rm tg}$ is fixed at 3.5 V. The average value of ΔR at the plateau, which measures the step height, saturates when $V_{\rm tg}$ is high enough (Fig. 1D, inset, and figs. S4 to S7).

This saturated value, ΔR_s , thus measures the resistance of the undoped channel, which can only originate from the edges because the monolayer interior is insulating (12-14). Notably,

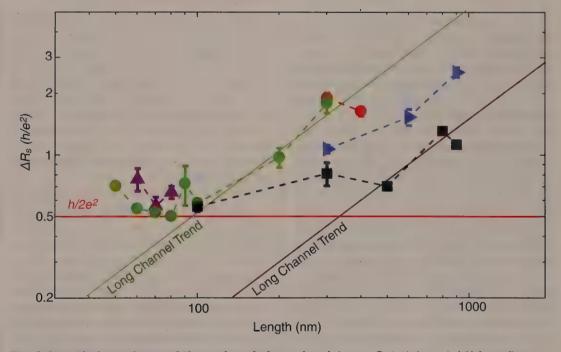
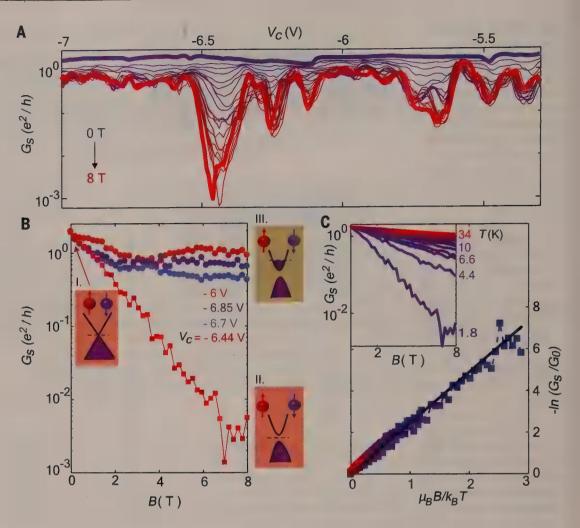


Fig. 2. Length dependence of the undoped-channel resistance. Data taken at 4 K from five different devices (table S1), each denoted by a different color and symbol. The device numbers and associated colors are: 1, black; 2, green; 3, purple; 4, red; and 5, blue. The ΔR_s values approach a minimum of $h/2e^2$ in the short-channel limit, confirming a total conductance of $2e^2/h$ for the undoped channel, i.e., a conductance of e^2/h per edge in the device, in agreement with QSHE. Detailed analysis of raw data can be found in figs. S4 to S7.

 ΔR_s is approximately equal to $h/2e^2$ for both this 100-nm channel and the 60- and 70-nm channels on device 2 (Fig. 1D). Fluctuations in the range of a few kilohm, which may originate from residual disorder or correlation effects (12, 27, 28), are visible, but decrease substantially above 4 K. Given that the sample has two edges, the observed conductance per edge is therefore $\sim e^2/h$, pointing to helical edge modes as the source of the conductance (5, 6). To confirm this scenario, one

Fig. 3. Magnetoconductance and Zeeman-like gap at the Dirac point. (A) The evolution of the edge conductance G_s versus local gate voltage V_c under the application of a perpendicular magnetic field, B (from 0 T, thick blue curve, to 8 T, thick red curve, in 0.2-T steps) at 1.8 K, for device 1, 100-nm channel. (B) Traces of Gs versus B for a few selected V_c , showing two types of behavior, saturation and nonsaturation, associated with whether the Fermi energy (E_F) is in the Zeeman gap, as depicted in the band schematics inset I (linear bands at zero B, EF at Dirac point), II (gapped bands at finite B; E_F at Dirac point), and III (gapped bands at finite B, E_F away from Dirac point). Red and blue spheres illustrate the opposite spin polarization of the edge bands, respectively. Purple areas indicate the filled bands. (C) Inset shows temperature dependence of G_s versus B for the nonsaturating curves ($V_c = -6.44 \text{ V}$). All the curves in the inset collapse to a single trend in the normalized plot of $-\log(G_s/G_0)$ versus $\mu_B B/k_B T$. The black line is a linear fit. Additional temperature and magnetic field dependence is shown in figs. S9 to S11.



must rule out the possibility of trivial diffusive edge modes that happen to exhibit the quantized conductance value for some particular length (22). We thus performed a length-dependence study using a series of local gates with different $L_{\rm c}$. Detailed analysis of measurements from representative devices and gates at ~4 K can be found in figs. S3 to S5. In Fig. 2, we summarize the data by plotting the undoped-channel resistance, ΔR_s , as a function of L_c . For long edges, the resistance generally decreases with decreasing length, which is arguably captured by a linear trend. The behavior, however, clearly deviates from the trend when L_c is reduced to 100 nm or less, where the resistance saturates to a value close to $h/2e^2$. Such behavior is present in all three devices that enter this short-length regime, independent of the width of the monolayer flake (varying from 1 to 4 µm). These observations reveal the intrinsic conductance as e^2/h per edge, as per the abovementioned criteria (i) and (ii) for the QSHE.

To check criterion (iii), regarding TR symmetry protection from elastic scattering, we performed magnetoconductance measurements. The data taken from the 100-nm-long channel in device 1 in the QSHE regime (i.e., gate range of the plateau) are shown in Fig. 3. We define G_s as 1/ $\Delta R_{\rm s}$, which measures the conductance of the edges in the short channel limit. G_s is plotted as a function of V_c in Fig. 3A for a series of magnetic fields B applied perpendicular to the monolayer at 1.6 K. Gs decreases substantially

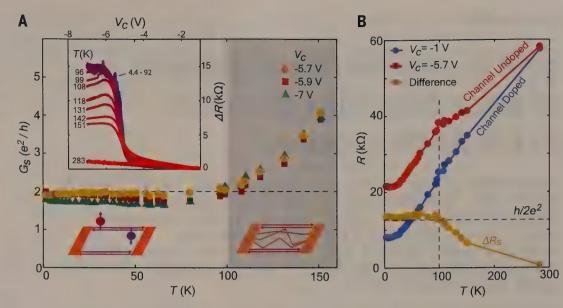
once B is turned on, in contrast to the bulk state, which is hardly affected (fig. S8). For all V_c , G_s decreases rapidly for low magnetic fields (B < 2 T). After this initial stage, two types of behavior are observed, depending on V_c , as shown in Fig. 3B. When V_c is near -6.44 V, G_s decreases exponentially without saturation, up to 8 T. For other values of V_c , G_s saturates at high B. These behaviors are notably different from the previous observations for resistive channels (12).

Both types of behavior can be understood in the context of the QSHE. The 1D edge state of the QSH phase consists of two species: left and right movers associated with opposite spin polarization. The two linearly dispersing bands cross at the Kramers degeneracy point (Fig. 3B, inset I). Magnetic fields applied nonparallel to the spin polarization are expected to open an energy gap at the Kramers point owing to the Zeeman effect (29). For a homogeneous chemical potential close to the degeneracy point (Fig. 3B, inset II), one would expect an exponential decay of the conductance without saturation. To reveal the existence of the gap, we performed temperaturedependence measurements of the magnetoconductance at V_c = -6.44 V. The exponential decay of G_s persists up to high temperatures (measured up to 34 K, inset of Fig. 3C). Moreover, all the curves collapse onto a single universal trend when renormalized by plotting the dimensionless values $-\log(G_{\rm s}/G_{\rm 0})$ versus $\mu_{\rm B}B/k_{\rm B}T$ (Fig. 3C), where G_0 is the zero-field conductance, μ_B is the Bohr magneton, k_B is the Boltzmann constant, and T is the temperature. The slope of the trend yields an effective g-factor ~4.8 for the out-ofplane field in this device [i.e., the device conductance obeys $G_s = G_0 \exp(-g\mu_B B/2k_B T)$]. This observation confirms a Zeeman-type gap opening in the edge bands.

If the Fermi energy at the edge is gated away from the Kramers degeneracy point (Fig. 3B, inset III), the Zeeman gap will not be directly observed, and the magnetoconductance should be determined by the scattering mechanisms at the edge allowed by the TR symmetry breaking. For example, in our devices, the presence of local charge puddles can be natural. According to theoretical calculations, the edge conductance will be reduced to $\alpha e^2/h$, where α is a fielddependent coefficient determined by the microscopic details of the edge (24, 30). Calculations show that, at high magnetic fields, an individual puddle can reduce transmission along an edge by 50% (24, 31), leading to a saturated α determined by the distribution of the puddles along the edges. We find the conductance saturation is consistent with this picture (fig. S9). In addition to vertical magnetic fields, we have also found considerably reduced edge conductance when inplane magnetic field is applied (fig. S10). We expect that both in- and out-of-plane magnetic fields will suppress the conductance: TR symmetry removes protection of the edge conduction, and the edge-mode spin-polarization axis is not necessarily normal or parallel to the layer because the

Fig. 4. Quantum spin Hall effect up to 100 K.

(A) Temperature dependence of the edge conductance at a few representative gate voltages for the 100-nm channel in device 1. The conductance is dominated by the OSHE up to about 100 K. The right schematic depicts the onset of bulk-state contribution to the conductance. Inset shows gate dependence of ΔR at various temperatures. (B) Temperature dependence of the resistance of the whole flake (full length) when the Fermi energy in the local channel is in the doped ($V_c = -1 \text{ V, red}$) and undoped ($V_c = -5.7 \text{ V,}$ blue) regimes, at $V_{\rm tg}$ = 3.5 V. The difference between the curves yields the temperaturedependent channel resistance ΔR_s (yellow). The vertical dashed line highlights the kink in the undoped regime at 100 K, indicating the transition to the QSHE edge-dominated regime.



monolayer lacks out-of-plane mirror symmetry. The exact spin-polarization axis may be influenced by multiple factors, such as the direction of the crystallographic edge and the existence of displacement electric fields. The irregular edge of the exfoliated monolayer makes the situation more complex. Overall, the magnetoconductance behavior is consistent with criterion (iii). Therefore, the QSHE is indeed observed in monolayer WTe2.

Notably, the distinctive zero-field conductance value survives up to high temperatures. Figure 4A plots the temperature dependence of G_s at different V_c in the QSHE regime; G_s stays approximately constant and close to $2e^2/h$ up to 100 K, indicating that the conductance is dominated by the QSHE up to this temperature. In terms of ΔR , the resistance plateau starts to drop at around 100 K (Fig. 4A, inset). We note that it is not obvious a priori what the temperature dependence of the QSH edge conductance should be, and some proposed mechanisms indicate weak (32) or even negative temperature dependence (27). Above 100 K, the channel conductance increases rapidly with temperature, indicating the activation of bulk-conduction channels. To reveal the transition more clearly, in Fig. 4B we plot the temperature dependence of the resistance R of the whole flake (i.e., the entire length, which consists of the locally gated region in series with the rest of the flake) when the chemical potential in the local channel is placed in the metallic regime ($V_c = -1 \text{ V}$) and the QSH regime (V_c < -5.3 V). A clear kink at 100 K can be seen in the QSH regime. The difference between the two curves yields the channel resistance, which drops above the transition temperature.

This high-temperature QSHE is consistent with the prediction of a large inverted band gap (~100 meV) in monolayer WTe₂ (20) as well as recent experiments that observe a ~45-meV bulk band gap in spectroscopy (13, 14) and a similar onset temperature for bulk conduction (12). We suspect the 100 K transition temperature may not be an intrinsic limit. Improvements in device quality may enable observation of the QSHE at even higher temperatures and for longer edges.

Our observations have confirmed the nontrivial TR invariant topological phase in monolayer WTe2 and demonstrated the QSHE at high temperatures in an isolated 2D monolayer device. The exploration of 2D topological physics and device performance above liquid nitrogen temperatures has therefore become possible. Distinct from quantum well systems, the exposed nature of isolated monolayers may allow for engineering topological phases in unprecedented ways. In particular, WTe2 can be readily combined with other 2D materials to form van der Waals heterostructures, a promising platform for studying the proximity effect between a QSH system and superconductors or magnets (3, 4) at the atomic scale.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/76/suppl/DC1 Materials and Methods Supplementary Text Figs. S1 to S11 Table S1 References (33, 34)

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CORAL REEFS

Spatial and temporal patterns of mass bleaching of corals in the Anthropocene

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Tropical reef systems are transitioning to a new era in which the interval between recurrent bouts of coral bleaching is too short for a full recovery of mature assemblages. We analyzed bleaching records at 100 globally distributed reef locations from 1980 to 2016. The median return time between pairs of severe bleaching events has diminished steadily since 1980 and is now only 6 years. As global warming has progressed, tropical sea surface temperatures are warmer now during current La Niña conditions than they were during El Niño events three decades ago. Consequently, as we transition to the Anthropocene, coral bleaching is occurring more frequently in all El Niño-Southern Oscillation phases, increasing the likelihood of annual bleaching in the coming decades.

he average surface temperature of Earth has risen by close to 1°C as of the 1880s (1), and global temperatures in 2015 and 2016 were the warmest since instrumental record keeping began in the 19th century (2). Recurrent regional-scale (>1000 km) bleaching and mortality of corals is a modern phenomenon caused by anthropogenic global warming (3–10). Bleaching before the 1980s was recorded only at a local scale of a few tens of kilometers because of small-scale stressors such as freshwater inundation, sedimentation, or unusually cold or hot weather (3–5). The modern emergence of regional-scale

bleaching is also evident from the growth bands of old Caribbean corals: synchronous distortions of skeletal deposition (stress bands) along a 400-km stretch of the Mesoamerican Reef have only been found after recent hot conditions, confirming that regional-scale heat stress is a modern phenomenon caused by anthropogenic global warming (10). Bleaching occurs when the density of algal symbionts, or zooxanthellae (Symbiodinium spp.),

distributed coral reef locations in 54 countries using a standardized protocol to examine patterns in the timing, recurrence, and intensity of bleaching episodes, including the latest global bleaching event from 2015 to 2016 (table S1). This approach avoids the bias of the continuous addition of new sites in open-access databases and retains the same range of spatial scales through time (fig. S1). A bleaching record in our analysis consists of three elements: the location, from 1 to 100; the year; and the binary presence or absence of bleaching. Our findings reveal that coral reefs have entered the distinctive human-dominated era characterized as the Anthropocene (16–18), in

in the tissues of a coral host diminishes as a re-

sult of environmental stress, revealing the underlying white skeleton of the coral (8). Bleached

corals are physiologically and nutritionally compromised, and prolonged bleaching over sev-

eral months leads to high levels of coral mortality (11, 12). Global climate modeling and satellite observations also indicate that the thermal con-

ditions for coral bleaching are becoming more prevalent (13, 14), leading to predictions that localities now considered to be thermal refugia could

Although several global databases of bleaching

records are available (notably ReefBase, reefbase.

org), they suffer from intermittent or lapsed main-

tenance and from uneven sampling effort across

both years and locations (7). The time spans of

five earlier global studies of coral bleaching range from 1870 to 1990 (3), 1960 to 2002 (4), 1973 to

2006 (5), 1980 to 2005 (6), and 1985 to 2010 (7).

Here we compiled de novo the history of recur-

rent bleaching from 1980 to 2016 for 100 globally

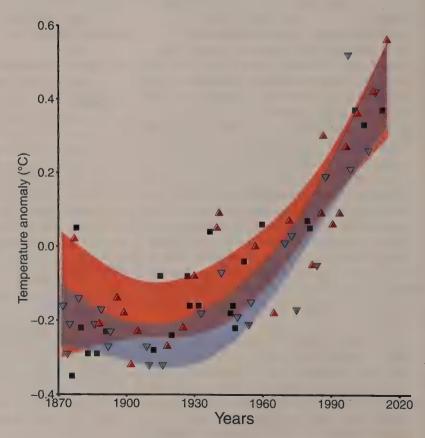
which the frequency and intensity of bleaching

events is rapidly approaching unsustainable levels. At the spatial scale we examined (fig. S1), the

disappear by midcentury (15).

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Fig. 1. Global warming throughout ENSO cycles. Sea surface temperature anomalies from 1871 to 2016, relative to a 1961-1990 baseline, averaged across 1670 1° latitudeby-1° longitude boxes containing coral reefs between latitudes of 31°N and 31°S. Data points differentiate El Niño (red triangles), La Niña (blue triangles), and ENSO neutral periods (black squares). Ninety-five percent confidence intervals are shown for nonlinear regression fits for years with El Niño and La Niña conditions (red and blue shading, respectively; overlap is shown in purple).



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number of years between recurrent severe bleaching events has diminished fivefold in the past four decades, from once every 25 to 30 years in the early 1980s to once every 5.9 years in 2016. Across the 100 locations, we scored 300 bleaching episodes as severe, i.e., >30% of corals bleached

at a scale of tens to hundreds of kilometers, and a further 312 as moderate (<30% of corals bleached). Our analysis indicates that coral reefs have moved from a period before 1980 when regional-scale bleaching was exceedingly rare or absent (3-5) to an intermediary phase beginning in the 1980s

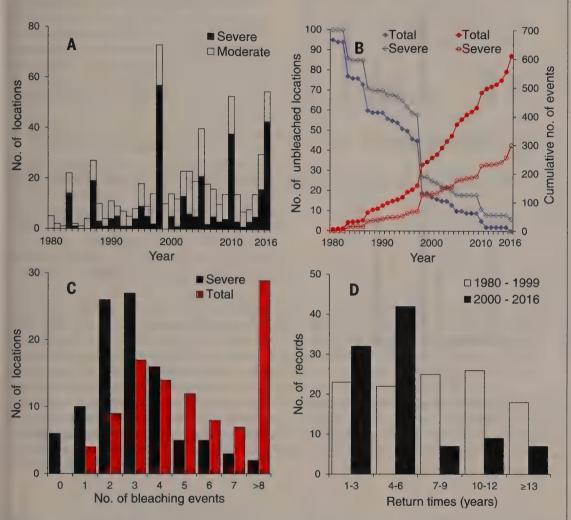


Fig. 2. Temporal patterns of recurrent coral bleaching. (A) Number of 100 pantropical locations that have bleached each year from 1980 to 2016. Black bars indicate severe bleaching affecting >30% of corals, and white bars depict moderate bleaching of <30% of corals. (B) Cumulative number of severe and total bleaching events since 1980 (red; right axis) and the depletion of locations that remain free of any bleaching or severe bleaching over time (blue; left axis). (C) Frequency distribution of the number of severe (black) and total bleaching events (red) per location. (D) Frequency distribution of return times (number of years) between successive severe bleaching events from 1980 to 1999 (white bars) and 2000 to 2016 (black bars)

when global warming increased the thermal stress of strong El Niño events, leading to global bleaching events. Finally, in the past two decades, many additional regional-scale bleaching events have also occurred outside of El Niño conditions, affecting more and more former spatial refuges and threatening the future viability of coral reefs.

Increasingly, climate-driven bleaching is occurring in all El Niño-Southern Oscillation (ENSO) phases, because as global warming progresses, average tropical sea surface temperatures are warmer today under La Niña conditions than they were during El Niño events only three decades ago (Fig. 1). Since 1980, 58% of severe bleaching events have been recorded during four strong El Niño periods (1982-1983, 1997-1998, 2009-2010, and 2015-2016) (Fig. 2A), with the remaining 42% occurring during hot summers in other ENSO phases. Inevitably, the link between El Niño as the predominant trigger of mass bleaching (3-5) is diminishing as global warming continues (Fig. 1) and as summer temperature thresholds for bleaching are increasingly exceeded throughout all ENSO phases.

The 2015-2016 bleaching event affected 75% of the globally distributed locations we examined (Figs. 2A and 3) and is therefore comparable in scale to the then-unprecedented 1997-1998 event, when 74% of the same 100 locations bleached. In both periods, sea surface temperatures were the warmest on record in all major coral reef regions (2, 19). As the geographic footprint of recurrent bleaching spreads, fewer and fewer potential refuges from global warming remain untouched (Fig. 2B), and only 6 of the 100 locations we examined have escaped severe bleaching so far (Fig. 2B and table S1). This result is conservative because of type 2 errors (false negatives) in our analyses, where bleaching could have occurred but was not recorded.

After the extreme bleaching recorded from 2015 to 2016, the median number of severe bleaching events experienced across our study locations since 1980 is now three (Fig. 2C). Eighty-eight percent of the locations that bleached from 1997 to 1998 have bleached severely at least once again. As of 1980, 31% of reef locations have experienced four or more (up to nine) severe

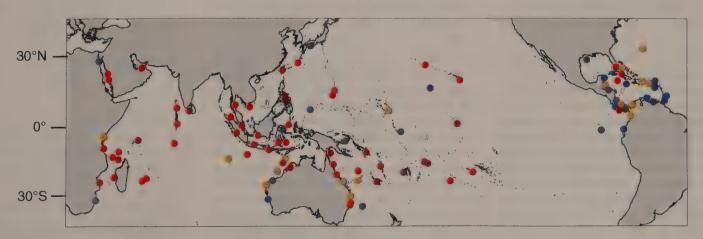


Fig. 3. The global extent of mass bleaching of corals in 2015 and 2016. Symbols show 100 reef locations that were assessed: red circles, severe bleaching affecting >30% of corals; orange circles, moderate bleaching affecting <30% of corals; and blue circles, no substantial bleaching recorded. See table S1 for further details.

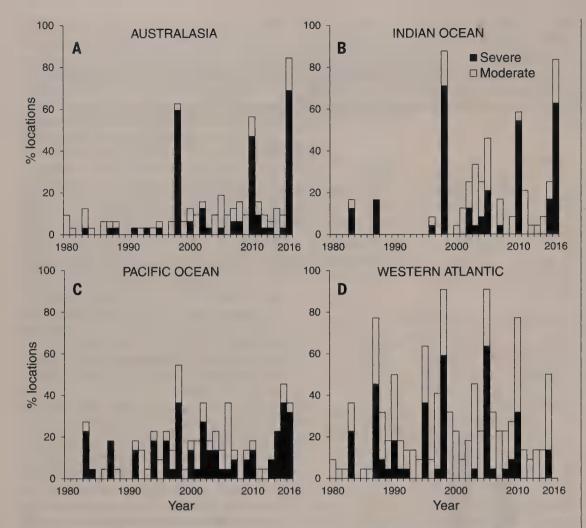


Fig. 4. Geographic variation in the timing and intensity of coral bleaching from 1980 to 2016.(**A**) Australasia (32 locations). (**B**) Indian Ocean (24 locations). (**C**) Pacific Ocean (22 locations).
(**D**) Western Atlantic (22 locations). For each region, black bars indicate the percentage of locations that experienced severe bleaching, affecting >30% of corals. White bars indicate the percentage of locations per region with additional moderate bleaching affecting <30% of corals.

bleaching events (Fig. 2C), as well as many moderate episodes (table S1). Globally, the annual risk of bleaching (both severe and more moderate events) has increased by a rate of approximately 3.9% per annum (fig. S2), from an expected 8% of locations in the early 1980s to 31% in 2016. Similarly, the annual risk of severe bleaching has also increased, at a slightly faster rate of 4.3% per annum, from an expected 4% of locations in the early 1980s to 17% in 2016 (fig. S2). This trend corresponds to a 4.6-fold reduction in estimated return times of severe events, from once every 27 years in the early 1980s to once every 5.9 years in 2016. Thirty-three percent of return times between recurrent severe bleaching events since 2000 have been just 1, 2, or 3 years (Fig. 2D).

Our analysis also reveals strong geographic patterns in the timing, severity, and return times of mass bleaching (Fig. 4). The Western Atlantic, which has warmed earlier than elsewhere (13, 19), began to experience regular bleaching sooner, with an average of 4.1 events per location before 1998, compared with 0.4 to 1.6 in other regions (Fig. 4 and fig. S2). Furthermore, widespread bleaching (affecting >50% of locations) has now occurred seven times since 1980 in the Western Atlantic, compared to three times for both Austra-

lasia and the Indian Ocean, and only twice in the Pacific. Over the entire period, the number of bleaching events has been highest in the Western Atlantic, with an average of 10 events per location, two to three times more than in other regions (Fig. 4).

In the 1980s, bleaching risk was highest in the Western Atlantic followed by the Pacific, with the Indian Ocean and Australasia having the lowest bleaching risk. However, bleaching risk increased most strongly over time in Australasia and the Middle East, at an intermediate rate in the Pacific, and slowly in the Western Atlantic (Fig. 4, fig. S3B, and tables S2 and S3). The return times between pairs of severe bleaching events are declining in all regions (fig. S3C), with the exception of the Western Atlantic, where most locations have escaped a major bleaching event from 2010 to 2016 (Fig. 2D).

We tested the hypothesis that the number of bleaching events that have occurred so far at each location is positively related to the level of postindustrial warming of sea surface temperatures that has been experienced there (fig. S4). However, we found no significant relationship for any of the four geographic regions, consistent with each bleaching event being caused by a short-

lived episode of extreme heat (12, 19, 20) that is superimposed on much smaller long-term warming trends. Hence, the long-term predictions of future average warming of sea surface temperatures (13) are also unlikely to provide an accurate projection of bleaching risk or the location of spatial refuges over the next century.

In the coming years and decades, climate change

will inevitably continue to increase the number of extreme heating events on coral reefs and further drive down the return times between them. Our analysis indicates that we are already approaching a scenario in which every hot summer, with or without an El Niño event, has the potential to cause bleaching and mortality at a regional scale. The time between recurrent events is increasingly too short to allow a full recovery of mature coral assemblages, which generally takes from 10 to 15 years for the fastest growing species and far longer for the full complement of life histories and morphologies of older assemblages (21-24). Areas that have so far escaped severe bleaching are likely to decline further in number (Fig. 2B), and the size of spatial refuges will diminish. These impacts are already underway, with an increase in average global temperature of close to 1°C. Hence, 1.5° or 2°C of warming above preindustrial conditions will inevitably contribute to further degradation of the world's coral reefs (14). The future condition of reefs, and the ecosystem services they provide to people, will depend critically on the trajectory of global emissions and on our diminishing capacity to build resilience to recurrent highfrequency bleaching through management of local stressors (18) before the next bleaching event occurs.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/80/suppl/DC1 Materials and Methods Figs. S1 to S4 Tables S1 to S3 References (25–29)

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ECOLOGICAL GENOMICS

Genomic signals of selection predict climate-driven population declines in a migratory bird

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The ongoing loss of biodiversity caused by rapid climatic shifts requires accurate models for predicting species' responses. Despite evidence that evolutionary adaptation could mitigate climate change impacts, evolution is rarely integrated into predictive models. Integrating population genomics and environmental data, we identified genomic variation associated with climate across the breeding range of the migratory songbird, yellow warbler (Setophaga petechia). Populations requiring the greatest shifts in allele frequencies to keep pace with future climate change have experienced the largest population declines, suggesting that failure to adapt may have already negatively affected populations. Broadly, our study suggests that the integration of genomic adaptation can increase the accuracy of future species distribution models and ultimately guide more effective mitigation efforts.

nthropogenic climate change is having a marked impact on Earth's biodiversity (1). Rapid fluctuations in temperature and precipitation can alter the suitability of particular regions and in some cases, exceed the physiological limits of organisms (2, 3). The mismatch between environment and physiology can lead to shifts in species ranges, population declines, or even extinction (4-6). The difference between these evolutionary outcomes can in part be determined by the adaptive capacity of a species; those that possess standing genetic variation for climate-related traits are most likely to have the ability to adapt to rapidly changing environments (7, 8). Understanding the effects and distributions of potentially adaptive alleles associated with climate can therefore aid efforts to

accurately predict species responses to future conditions (9), and aid in mitigation efforts.

Although migratory birds are highly sensitive to climate change (10-12), little is understood about how populations differ in adaptive capacity. Breeding ranges of North American migrants have shifted northward (13), but microevolutionary responses associated with these shifts remain poorly documented. Although genome-wide associations with climate variables have been shown to affect many groups across the tree of life (14-16), in migratory species it is less clear to what extent genomic variation is shaped by environmental variables. Here, we examined the genomic basis of climate adaptation in a North American migratory bird, the yellow warbler (Setophaga petechia). Yellow warblers have a broad breeding range across the United States and Canada and are common throughout their range, though they have experienced local population declines and, in some regions, are listed as a species of concern (17). Because of their broad distribution, yellow warblers inhabit a large range of environmental conditions, making them an ideal system for investigating variation in local climate adaptation.

We used restriction site-associated DNA sequencing (RAD-Seq) to test for signals of selection across the breeding range. We examined 104,711 single-nucleotide polymorphisms (SNPs)

in 229 individuals from 21 locations (table S1 and fig. S1), using our assembly of the first yellow warbler genome (18). Pairwise genetic distance between locations $(F_{ST}/1 - F_{ST})$ was highly correlated with geographic distance, suggesting a strong signal of isolation by distance (Fig. 1A: Mantel's r = 0.85, $P = 1 \times 10^{-5}$), consistent with previous findings using microsatellite loci (19). Analysis of population structure using ADMIXTURE found little evidence of substructure, consistent with strong isolation by distance (fig. S2). Genetic distance was also significantly associated with environmental distance, based on climate variables, vegetation indices, and elevation, downloaded from public environmental databases (Fig. 1B: Mantel's r = 0.36; P = 0.0006). In a multiple regression of distance matrices, only geographic distance was significant (MRM: $R^2 = 0.73$; geography $P = 1 \times 10^{-5}$; environment P = 0.12), suggesting that isolation by distance is the strongest force structuring genome-wide variation. This result is not unexpected, however, because environmental adaptation likely affects a small fraction of the genome (9). We used gradient forest (20), a machine-learning regression tree-based approach, to test whether a subset of genomic variation can be explained by environment and to visualize climate-associated genetic variation across the breeding range (Fig. 1, C and D). Strong differences in environmentally associated genetic variation are apparent across longitude and latitude, and unique genotype-environment associations are present in the Rocky Mountains and Coastal British Columbia. Our results suggest that despite high dispersal capacity and vagility via annual migration, yellow warblers exhibit standing genetic variation associated with the environment among populations and are likely subject to environmentally mediated selection during the breeding season.

To investigate which populations might be most vulnerable to future climate change, we defined the metric "genomic vulnerability" as the mismatch between current and predicted future genomic variation based on genotype-environment relationships modeled across contemporary populations. We followed the method presented in Fitzpatrick and Keller (21) to calculate genomic vulnerability using an extension of the gradient forest analysis. Populations with the greatest mismatch are least likely to adapt quickly enough to track future climate shifts, potentially resulting in population declines or extirpations. Under future climate change scenarios [representative

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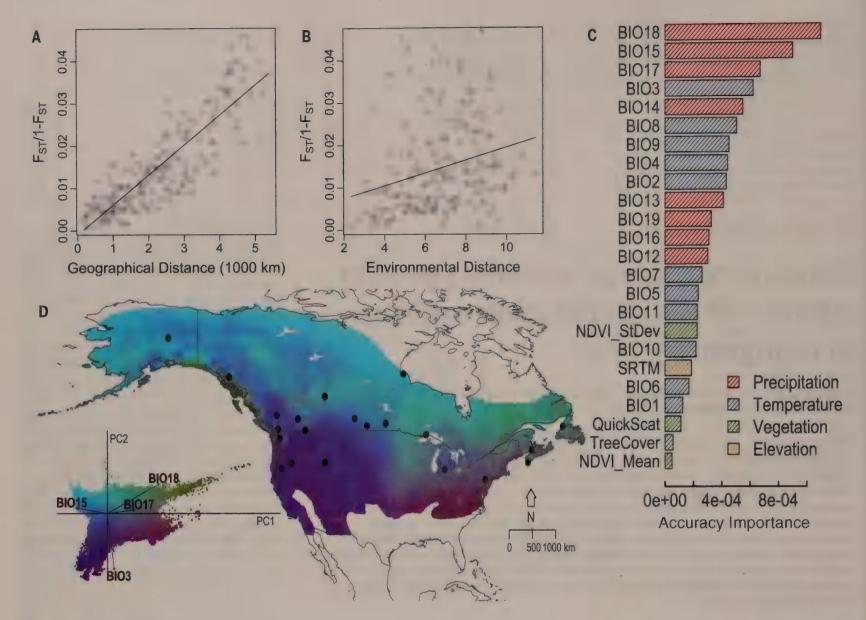


Fig. 1. Geography and environment explain genomic variation in yellow warblers. (A) Pairwise genetic distance $(F_{\rm ST}/1-F_{\rm ST})$ is associated with geographical distance and (B) environmental distance. (C) Ranked importance of environmental variables based on gradient forest analysis shows that climate, especially precipitation, strongly explains genomic variation.

(**D**) Gradient forest-transformed climate variables show climate adaptation across the breeding range. Colors are based on principal components analysis (PCA) of transformed climate variables [(D) inset: loadings of all variables are shown in fig. S3]. Points on map reflect sampled locations, and arrows on PCA show the loadings of top climate variables on PCA.

concentration pathways—RCPs—defined by the Intergovernmental Panel on Climate Change (22)], the regions with highest genomic vulnerability stretch from the southern Rocky Mountains to Alaska, along with patchy regions in the Eastern United States (Fig. 2A). As expected, genomic vulnerability increases under more severe climate change scenarios; under the most extreme scenario (RCP8.0), nearly the entire range is estimated to have high genomic vulnerability (fig. S4).

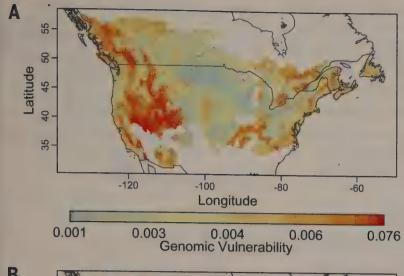
If future climate change is correlated with recent shifts (for example, if regional drying over the last century in some regions will continue and become more severe), we expect that recent climate change will have already negatively affected populations with high genomic vulnerability. We tested this idea by comparing genomic vulnerability scores (using 2050 RCP2.6, though the results were robust to different scenarios; fig. S4) to population trends estimated from North American Breeding Bird Surveys (23) (Fig. 2B). Regions that had higher genomic vulnerability scores experienced the largest population de-

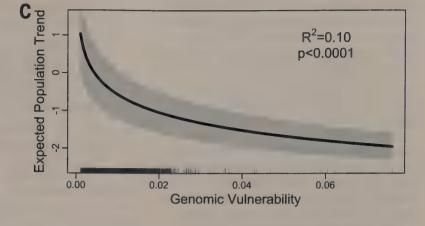
clines over the past half century [generalized additive model (GAM): adjusted $R^2 = 0.101$; P < 0.001; Fig. 2C], showing that populations already in decline are most vulnerable to future climate change and suggesting that a mismatch between genomic variation and climate may have already resulted in negative impacts. We believe that genomic vulnerability can therefore be used alongside other known causes of population decline, such as habitat degradation and avian disease, to understand and predict population dynamics.

Understanding which environmental variables are most closely associated with local adaptation to particular climate conditions and vulnerability can prove useful in determining the biological mechanisms involved in population declines. Climate variables, especially precipitation measures, were most strongly associated with genomic variation across the breeding range of yellow warblers (Fig. 1C). Of the 25 environmental variables tested in the gradient forest analysis, the top three explanatory variables were precipitation related: (i) precipitation of the warmest

quarter (BIO18), (ii) seasonality of precipitation (BIO15), and (iii) precipitation of the driest quarter (BIO17). Overall, precipitation variables were most important, followed by temperature variables and to a lesser extent, vegetation and elevation variables, suggesting that adaptation to precipitation is important on the breeding grounds.

To investigate the genomic basis of adaptation across contemporary climate gradients, we identified genomic regions associated with the top precipitation-related variables using latent factor mixed models (LFMMs) (24), which test for associations between genotypes and environments while accounting for background population structure (Fig. 3A and fig. S5). We found 85, 67, and 35 SNPs associated with climate variables BIO18, BIO15, and BIO17 (described above), respectively [false discovery rate (FDR)–corrected P < 0.05]. These SNPs were broadly distributed across the genome, on 27 chromosomes (table S2). For the 13 SNPs associated with all three variables, we identified 12 adjacent genes with a range of





Genomic Vulnerability

B

99

-120

-100

Longitude

-2

Population Trend (% change)

Fig. 2. Genomic vulnerability to future climate change is associated with ■ higher probability of population decline. (A) Genomic vulnerability based on 2050 RCP2.6 projections. (B) Population trend estimates (percent change per year) for yellow warblers based on North American Breeding Bird Survey analysis (23). (C) Generalized additive model (GAM) showing the relationship between population trend and predicted genomic vulnerability. The black line represents the model fit, and the shaded area is the 95% confidence interval.

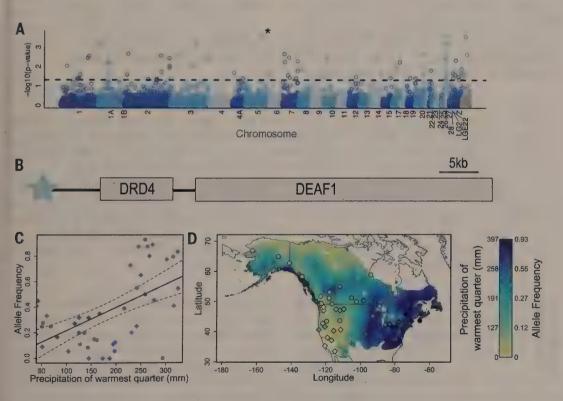


Fig. 3. Genome-wide variation associated with climate variables. (**A**) Manhattan plots
show the significance level (FDR-corrected) for
SNP associations with precipitation of the
warmest month (BlO18). Dashed line represents P = 0.05. Colors distinguish different chromosomes, and gray points are SNPs on scaffolds
not anchored to a chromosome. (**B**) The most
significant SNP association, marked with an
asterisk (*) in (A), is upstream from the *DRD4*and *DEAF1* genes. (**C** and **D**) Correlations
between allele frequency and BlO18 for this
SNP. Samples genotyped by RAD-Seq are
represented as circles, and samples genotyped
with Fluidigm assays are shown as diamonds.

functions including zinc finger genes (*ZNF397* and *ZKscan1*), peptide secretion (*SCT* and *MUC4*), and transmembrane proteins (CDHR5, SLC25A33, and TEMEM201). Targeted genotyping using Fluidigm assays for 17 SNPs associated with climate in the LFMM analysis in an additional 309 birds at 29 locations independently validated climate

associations in 8 out of 17 SNPs (FDR-corrected P < 0.05; table S3) with marginal associations in an additional two SNPs (FDR-corrected P < 0.1).

One of the strongest associations between genotype and climate was upstream of genes with known function in avian behavior and migration. A SNP on chromosome 5 was very strongly associated with all three top environmental variables (LFMM P < 0.001; Fig. 3), and these associations were validated with Fluidigm assays (P < 0.001; Fig. 3, C and D). The highest allele frequencies at this SNP occurred in the Maritime provinces of Canada (Nova Scotia and Newfoundland), areas of high rainfall and low seasonality. This SNP is

upstream of two genes, DRD4 and DEAF1 (Fig. 3B), that have known associations with migration in birds (25, 26). The DRD4 gene in particular, a dopamine receptor, has been extensively studied for its involvement in novelty-seeking behavior in primates, fish, and birds (26). Polymorphisms in this gene are linked to novelty-seeking or exploratory behavior in a number of bird species (27, 28), and linkage blocks extend into the neighboring DEAF1 gene, a transcription factor involved in serotonergenic signaling. The exploratory phenotype has been linked to dispersal, which is thought to allow species to occupy new environments (25), but experimental studies linking behavior, genotype, and environment are needed to fully understand how these variants might be involved in climate adaptation. However, this SNP represents a single locus with significant associations between genotype and environment, but many other highly significant candidates exist (table S2), highlighting the complex and polygenic nature of climate adaptation.

Expected patterns of environmentally mediated evolution in migratory animals are complex but could drastically alter predictions of species response to climate change. We showed that standing variation for adaptation to different climate regimes exists and that natural selection during breeding months is driving evolutionary shifts in the genome. High genomic vulnerability, or mismatch between current allelic variation and future environmental conditions, was correlated with a higher likelihood of population decline, suggesting that yellow warbler populations may have already experienced some negative impacts of climate change over the past 50 years. Our results show how the integration of genomic, environmental, and demographic data can provide a more thorough understanding of future climate change impacts on a migratory bird species. More broadly, we illustrate a new approach for understanding climate-associated causes of current and future declines and an important tool for making more-informed conservation decisions.

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SUPPLEMENTARY MATERIALS

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HIV SUSCEPTIBILITY

Elevated *HLA-A* expression impairs HIV control through inhibition of **NKG2A-expressing cells**

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The highly polymorphic human leukocyte antigen (HLA) locus encodes cell surface proteins that are critical for immunity. HLA-A expression levels vary in an allele-dependent manner, diversifying allele-specific effects beyond peptide-binding preference. Analysis of 9763 HIV-infected individuals from 21 cohorts shows that higher HLA-A levels confer poorer control of HIV. Elevated HLA-A expression provides enhanced levels of an HLA-A-derived signal peptide that specifically binds and determines expression levels of HLA-E, the ligand for the inhibitory NKG2A natural killer (NK) cell receptor. HLA-B haplotypes that favor NKG2A-mediated NK cell licensing (i.e., education) exacerbate the deleterious effect of high HLA-A on HIV control, consistent with NKG2A-mediated inhibition impairing NK cell clearance of HIV-infected targets. Therapeutic blockade of HLA-E:NKG2A interaction may yield benefit in HIV disease.

iversity within regions of human leukocyte antigen (HLA) class I molecules that determine peptide-binding specificity has a major impact on human disease pathogenesis. Variation in expression levels across alleles of certain HLA genes has also been shown to associate with disease outcome (1-6), emphasizing the importance of HLA polymorphism that determines characteristics other than peptide specificity alone. Elevated expression levels of HLA-C associates with reduced HIV viral load (VL) (1), resulting, in part, from a greater frequency of cytotoxic T lymphocyte (CTL) responses to HLA-Crestricted peptides with increasing HLA-C. Like HLA-C, HLA-A alleles vary in expression levels in an allotype-specific manner (7), but these two class I loci have many distinguishing characteristics. Compared with HLA-C, HLA-A is expressed at a 13- to 18-fold higher level on the cell surface (8) and is about twofold more polymorphic. Mechanisms of transcriptional regulation for these two loci are also distinct under healthy conditions (7, 9, 10). These and other differences may affect how these two loci affect human disease.

We verified that the pattern of allele-specific variation in HLA-A expression levels was not modified by HIV infection by comparing HLA-A expression in 243 HIV-uninfected and 162 HIVinfected ethnicity-matched individuals (fig. S1). Being HIV infected did not associate with a change

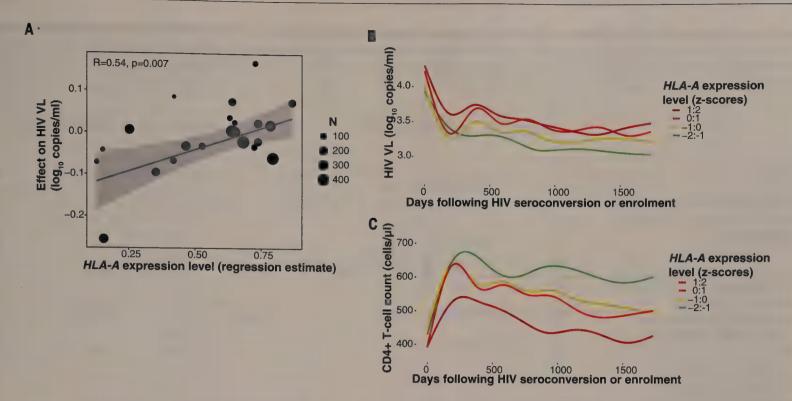


Fig. 1. Elevated *HLA-A* expression levels are associated with increased HIV viremia and reduced CD4⁺ T cell counts. (A) Data represent 2298 HIV-infected individuals from South Africa, Botswana, and Zambia, enrolled at 11 sites with cross-sectionally measured VLs. Each dot represents the average estimated expression level for a specific *HLA-A* allele by that allele's reported effect on cross-sectional VL (11). A linear regression line is shown in blue with 95% confidence interval in gray. The

size of each point is scaled by the number of contributing alleles; however, the correlation estimate is not weighted. (**B**) HIV viremia among 5818 HIV-infected adults and (**C**) CD4⁺ T cell counts among 2100 HIV-infected adults followed prospectively and grouped according to one-unit z-score change in HLA-A expression. VLs are plotted against time following seroconversion or date of enrollment (censored at ~5 years). In (B) and (C), lines are best fit (LOWESS lines) to unadjusted VL or CD4 counts.

in the overall level of HLA-A mRNA expression (Effect_{unadjusted} = 0.00, SE = 0.07, P =1), nor did HIV status modify expression estimates for any single HLA-A allele (interaction P-values were 0.226 to 0.987 for each of the alleles tested). Therefore, in HIV infection, the gradient in HLA-A expression level attributable to each allele is similar to that in healthy individuals.

To test whether HLA-A expression levels are associated with HIV control, we examined a pooled data set of 2298 HIV-infected (clade C) individuals recruited at 11 sites in sub-Saharan Africa, in which the estimated effect of each HLA allele on HIV VL measured cross-sectionally has been reported (II). The HLA-A expression level of each allele, estimated for black African individuals, was positively correlated with the estimate of effect of that allele on HIV VL (correlation coefficient R = 0.54, P = 0.007, Fig. 1A and Table 1).

Next, we sought to validate the discovery of a deleterious effect of elevated *HLA-A* expression

level in independent cohorts with prospective follow-up and of broader demographic background. We included 62,843 VL measurements obtained longitudinally over a total of 32,804 person years of antiretroviral therapy-free observation time (median 2.86 years per individual) in 5818 individuals enrolled in one of six studies in the USA or one study in Switzerland (see online methods). We modeled HLA-A expression as z-scores (equivalent to one standard deviation change in expression level), using mRNA levels measured in 436 white and black healthy donors (table S1). Consistent with the discovery analysis among sub-Saharan Africans, elevated HLA-A expression levels were significantly associated with higher HIV viremia, even after accounting for the individual allelic effects of HLA-A, -B, and -C. For every one z-score increase in HLA-A expression level, the VL increase over time was $0.06 \log_{10} \text{ copies/ml higher } (P = 4.4 \times 10^{-19});$ Table 1). Grouping individuals by estimated HLA- A expression level demonstrates the effect of increasing *HLA-A* expression on unadjusted HIV VL (Fig. 1B).

The association between HLA-A expression level and HIV viremia was independently significant in each ethnicity stratum ($P_{\text{whites}} = 6.1 \times$ 10^{-6} ; $P_{\text{Africans/African-Americans}} = 1.1 \times 10^{-18}$; and $P_{\text{Hispanic/other}} = 2.3 \times 10^{-10}$), notwithstanding distinct HLA-A allelic frequencies in each ethnic group. Among 2019 donors enrolled during acute, early HIV infection with known dates of seroconversion, elevated HLA-A expression was similarly associated with higher VL ($P = 2.5 \times 10^{-9}$), confirming that this finding is unlikely to be confounded by frailty bias. HLA-A expression level was associated with a spectrum of alternative HIV outcomes, including elevated mean VL ($P = 9.3 \times$ 10⁻¹²) and odds of being an HIV noncontroller (HIV VL >10,000 copies/ml) relative to being a controller (HIV VL < 2000 copies/ml) ($P = 9.2 \times$ 10⁻¹¹). Furthermore, among 2100 individuals for

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Table 1. *HLA-A* expression level is associated with impaired HIV control and is robust to multiple outcome definitions, and subset analyses across 9763 independent individuals of varying geographic and ethnic background. Effect estimates denote the effect of one z-score (i.e. one standard deviation) increase in *HLA-A* expression on the outcome denoted.

Study	Outcome measure	Modeling approach	n	Effect estimate per <i>HLA-A z-</i> score increase	95% CI	P-value
	Cr	oss-sectional discovery	/ studies			
Pooled analysis of 2298 individuals from 11 African sites (11). Black individuals only.	Viral load (log ₁₀ copies/ml)	Spearman correlation of VL effect and expression level for 23 HLA-A alleles	2298 volunteers	Spearman R = 0.54	NA [‡]	0.007
	Prospective va	alidation studies with lo	ngitudinal follow-up			•••••
Pooled analysis of 581/ individuals	Longitudinal viral	Mixed effects-linear*				***************************************
from six U.S. cohorts (ACTG,	load (VL)					
ALIVE, MACS, MHRP, Ragon,	All individuals		62,843 VL in 5,818	0.06 log ₁₀	0.05:0.08	4.4×10^{-19}
SCOPE) and one Swiss cohort			volunteers	copies/ml		
(SWISS). Pooled data from	Known date of	***************************************	21,817 VL in 2,019	0.06 log ₁₀	0.04:0.08	2.5×10^{-9}
3442 white, 1497 black,	seroconversion		volunteers	copies/ml		
233 Hispanic, 60 Asian,						
14 other, and 572 of mixed						
or other ancestry. Mean viral load (mVL)	Mixed effects-linear*	5,818 mVL in 5.818 volunteers	0.14 log ₁₀ copies/ml	0.10:0.18	9.3 × 10 ⁻¹²	
Controller/non-controller	Mixed effects-binomial*		OR [§] = 1.30	1.20:1.42	9.2 × 10 ⁻¹¹	
CD4 ⁺ T cell count (cells/μl)	Mixed effects-linear*	56,415 CD4 counts in 2,100 volunteers	-37.8 cells/µl	-41.3:34.2	5.9 × 10 ⁻⁹⁴	
	Prospec	tive natural history val	dation studies			***************************************
Pooled analysis of 1159 individuals from five U.S. sites (ALIVE,	Time to AIDS (CDC 1987)	Mixed effects-Cox*	1159 at-risk individuals, 433 events	HR ^{II} = 1.25	1.01:1.55	0.04
MACS, MHCS, SFCCC and DCGCS). Pooled data from						
white, black, Hispanic or						
other ethnicities. Time to CD4 <200 cells/μl	Mixed effects-Cox*	1159 at-risk individuals, 537 events	HR = 1.24	1.03:1.49	0.02	
	Reanalysis of broad	HIV case-control geno	me-wide association st	udy	******	*****
Pooled analysis of 3057 white, Hispanic, and black ethnicities.	Controller/ non-controller	Logistic-regression [†]	737 controller/ 2300 noncontroller	OR = 1.29	1.14:1.45	2.7 × 10 ⁻⁵

*HLA-A, -B, and -C alleles, and timing of viral load measurements (for prospective studies) were taken into account by being coded as random effects.

†For GWAS analysis, population structure was adjusted for using the top five principal components.

‡NA, not applicable.

§OR, odds ratio.

||HR, hazard ratio.

whom longitudinal CD4⁺ T cell count measures were available, higher HLA-A expression was strongly, and substantially, associated with reduced CD4+ T cell counts (Table 1). The effects of HLA-A expression levels on VL and CD4 count were stable over time (Fig. 1, B and C), consistent with a temporally sustained mechanism. Finally, we examined a partially nonoverlapping (39.1% of donors were not included in the VL analyses) collection of five naturalhistory cohorts, including 1159 antiretroviral-naïve individuals followed prospectively after HIV infection. Even in this limited sample, elevated HLA-A expression was associated with accelerated progression to AIDS₁₉₈₇ (P = 0.04) and progression to $CD4^{+}$ T cell count of <200 cells/ μ l (P = 0.02), again after adjusting for all individual HLA alleles.

HLA-A expression levels vary across alleles in a continuous manner, indicating multiple polymor-

phic regulatory sites that together determine the expression level of any given allele. As no single variant controls HLA-A expression levels, genomewide association studies (GWAS) are not expected to detect such effects. Using formal HLA-A typing results, we inferred expression level for 3057 white, Hispanic, and black individuals included in the International HIV Controllers GWAS (12) (40% of whom were not included in any of the analyses described above). HLA-A expression was significantly associated with HIV elite controller or noncontroller status even after adjusting for population structure ($P = 2.7 \times 10^{-5}$). This observation emphasizes a limitation of GWAS when the combined effects of multiple genetic variants determine a phenotype.

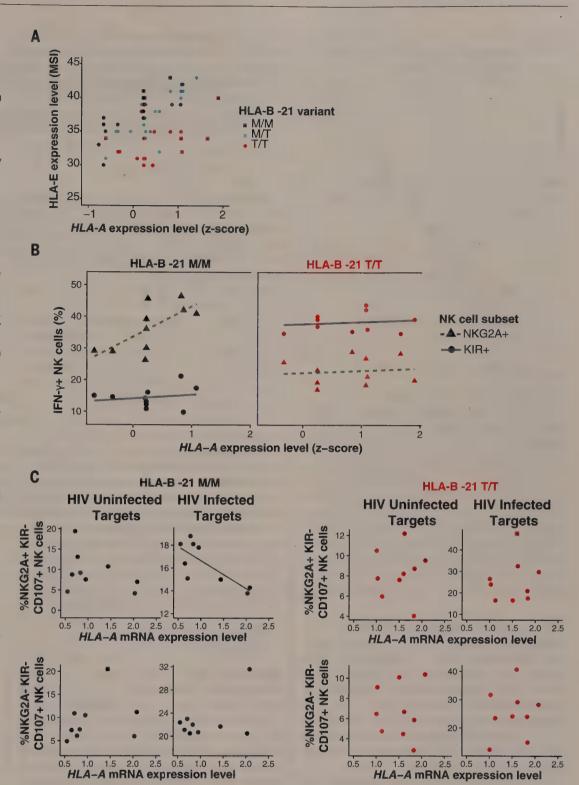
Next, we sought to determine the likely mechanism(s) for the finding that elevated *HLA-A*

expression associates with impaired HIV control. HLA-E serves as a ligand for the strongly inhibitory receptor CD94/NKG2A expressed on both natural killer (NK) cells and T cells. Expression of HLA-E is dependent on stable binding of a signal peptide derived from the leader sequence of HLA-A, -B and -C molecules (residues -22 to -14 relative to the mature protein) (13, 14). Methionine at position 2 of the signal peptide (residue -21) stabilizes and promotes HLA-E expression, and all HLA-A and -C allotypes are fixed for methionine, whereas HLA-B contains a polymorphism that encodes either methionine (-21M) or threonine (-21T) at this position (15, 16). Unlike HLA-A, there is minimal variance in HLA-B transcriptional levels across alleles and individuals (17), so HLA-E expression is expected to vary not as a consequence of differences in HLA-B expression

Fig. 2. HLA-A expression and HLA-B -21M regulate HLA-E expression, resulting in biased licensing of NKG2A-expressing NK cells that are impaired in their killing of HIV-infected target cells. (A) HLA-E expression according to HLA-A expression and HLA-B -21M in 58 HIV-uninfected donors. Each dot represents HLA-E expression levels (expressed as median signal intensity on a linear scale), as determined by CyTOF (15), and imputed HLA-A expression (z-score) ($R_{pearson} = 0.43$; 95% CI 0.20-0.62; P = 5×10^{-4}). (**B**) NKG2A⁺ NK cell licensing varies by HLA-A expression and HLA-B -21M. Peripheral blood mononuclear cells (PBMCs) from 10 HLA-B -21M/M and 10 HLA-B -21T/T donors were coincubated with Raji cells pretreated with mouse antibody (2.5 µg/ml) against human CD20 for 6 hours to probe NK cell licensing and education. Each point represents the proportion of IFN-y+ NK cells from each individual that are NKG2A+/KIR (triangles) or KIR⁺/NKG2A⁻ (circles) as a function of HLA-A expression. Dotted and solid lines show best fit lines for NKG2A+ and KIR+ subsets. respectively. The association between NK cell responsiveness and HLA-A expression for NKG2A+ NK cells in HLA-B -21M/M donors was R_{pearson} = 0.69 (95% CI 0.10-0.92), P = 0.03; all other correlations were not significant. (C) PBMCs from 9 HLA-B -21M/M and 9 HLA-B -21T/T donors were cocultured for 6 hours with autologous T cell blasts that were left uninfected or were infected with HIV [vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped NL4-3] and stained for CD107A, a marker of NK cell degranulation (see fig. S3 for gating strategy). HLA-A expression was formally measured in these T cell blasts by quantitative polymerase chain reaction and is expressed relative to β2M expression levels. Plots show individual proportions of NK cells expressing CD107a among NKG2A+KIR- and NKG2A-KIRsubsets. A best fit line is shown for significantly correlated observations. Red and black lines and dots denote TT and MM donors, respectively. The association between NKG2A+KIR- NK cell response to HIV-infected target cells, and HLA-A expression in HLA-B -21M/M donors was R_{pearson} = -0.77 (95% CI -0.21 to -0.95), P = 0.02; all other correlations were not significant.

levels, but rather as a result of HLA-B -21M/T variation. Accordingly, HLA-B -21M enhances HLA-E expression level in a copy-dependent manner (*15*). We tested whether *HLA-A* expression levels may similarly be associated with HLA-E expression levels. Among 58 healthy donors, higher predicted *HLA-A* expression levels, and therefore higher HLA-A-derived signal peptide, was significantly correlated with higher HLA-E expression levels on the cell surface, independently of the reported effects of HLA-B -21 (Fig. 2A and table S2).

HLA-E has two common allelic variants denoted E*01:01 and E*01:03, reportedly varying in peptide affinities, peptide repertoires, and surface expression levels (18). Although HLA-E*01:03 associates with higher surface expression in univariate analyses, this association was not significant after ad-



justing for HLA-B -21 and HLA-A genotypes (table S2). As HLA-E*01:03 and HLA-B -21M alleles are in significant linkage disequilibrium (D' = 0.52), the increased peptide supply attributable to HLA-B -21M and HLA-A expression level likely account for higher expression of HLA-E*01:03, rather than the variant distinguishing HLA-E*01:03 from -E*01:01. Accordingly, HLA-E variants did not show independent association with HIV outcomes (table S3). Similarly, addition of HLA-E genotype to a model fitting HLA-A expression and HLA-B -21M (and their interaction) was inferior to a model excluding HLA-E genotype in explaining HIV viremia.

The responsiveness of NK cells varies according to the presence of inhibitory-receptor/HLA pairs because of a process termed NK cell education or licensing (19). Accordingly, quantitative

variation in HLA expression may influence target cell recognition through both ligand density variation and licensing modulation. The HLA-B -21 M/T variant distinguishes between two sets of HLA haplotypes that have differential effects on NK cell education, where -21M marks haplotypes that bias toward NKG2A-mediated education and -21T marks alternative haplotypes that bias toward KIR (killer cell immunoglobulinlike receptor)-mediated education (15). The reported linkage disequilibrium between HLA-B -21M and HLA-B Bw6/HLA-C group1 alleles that interact poorly with KIR is evident in our cohort (fig. S2). Using a ligand-independent activation assay designed to measure NK cell licensing, NKG2A⁺/KIR⁻ NK cells from *HLA-B -21MM*+ donors were more responsive than NKG2A⁻/KIR⁺ NK cells from the same donors ($P_{\text{wilcoxon}} = 1.5 \times 10^{-6}$), and notably,

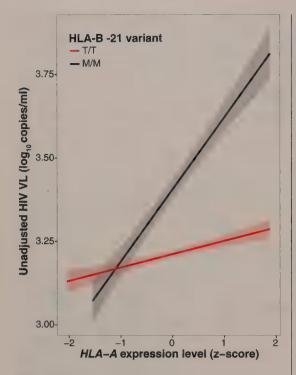


Fig. 3. The effect of HLA-A expression un HIV VL is modified by HLA-B alleles encoding methionine at position -21 in the signal peptide. The magnitude of effect (slope) of HLA-A expression on HIV viral load is stronger among individuals with HLA-B -21 MM (VL from 428 individuals, black line, VL_{effect-MM} = 0.22 log₁₀ copies/ml, $P = 1.5 \times 10^{-21}$ adjusted for HLA-A, -B, and -C) compared with HLA-B TT (VL from 3071 individuals, red line, VL_{effect-TT} = 0.06 log₁₀ copies/ml, $P = 1.8 \times 10^{-9}$ adjusted for HLA-A, -B, and -C). Interaction $P = 5.3 \times 10^{-9}$. Gray shading represents 95% CI of the linear estimate.

the strength of licensing among NKG2A+/KIR-NK cells correlated with HLA-A expression level (R = 0.69, P = 0.03; Fig. 2B). Conversely, KIR⁺/ NKG2A NK cells were more strongly licensed in HLA-B –21TT donors ($P = 1.1 \times 10^{-5}$), and this was not correlated with HLA-A expression. Thus, HLA haplotypes characterized by both HLA-B -21M and high HLA-A genotypes, which provide highest levels of HLA-E epitope, strongly bias toward NKG2A-mediated education.

We next tested whether variation in HLA-A expression alters NK cell responses toward HIVinfected target cells, and whether this varies according to HLA-B -21 genotype. Increasing HLA-A expression was significantly correlated with greater inhibition of NK cell degranulation exclusively among HLA-B -21MM donors, when target cells were HIV infected and the autologous effector NK cells necessarily expressed NKG2A (R = -0.77, P = 0.016, Fig. 2C). These data extend previous observations (20).

We reasoned that the genetic epidemiological effect of HLA-A expression level on impairing HIV control may vary according to HLA-B-21 genotype. We examined the two extremes in variation of NK cell education demarcated by *HLA-B* –21 MM versus TT, although education varies across a continuum (21). Haplotypes tagged by HLA-B -21M exacerbate the deleterious effect of HLA-A expression on HIV viremia (interaction $P = 5.3 \times 10^{-9}$), regardless of ethnicity (Fig. 3). The effect of HLA-A expression level on HIV viremia is of greater magnitude in individuals with two HLA-B methionine-encoding alleles [VL_{effect-MM} = 0.22, 95% confidence interval (CI) 0.17-0.26 log₁₀ copies/ml per one z-score, $P = 1.5 \times 10^{-21}$] than in donors with two threonine-encoding HLA-B alleles ($VL_{effect-TT} = 0.06, 95\% \text{ CI } 0.04-0.08 \log_{10}$ copies/ml per one z-score, $P = 1.8 \times 10^{-9}$). The independent effect of HLA-B -21M varied across Caucasians and Africans/African Americans (fig. S4), perhaps owing to substantial differences in HLA haplotypes in Africans. In an HLA-B-21M/M individual, decrease in HLA-A expression by two z-scores (0.44log₁₀ copies/ml lower VL) is comparable in magnitude to the effect of the presence of HLA-B*57 (0.41 log₁₀ copies/ml lower VL in the same data set).

Taken together, these data support a model of increased HLA-A expression having a deleterious effect on HIV control through enhanced HLA-E expression that results in increased NKG2Amediated NK (and/or T cell) inhibition, and impaired elimination of HIV-infected target cells.

HIV is capable of avoiding both T cell and NK cell recognition of infected host cells. HIV Nefmediated reduction of HLA-A and -B (22) surface expression and Vpu-mediated reduction of HLA-C (23) likely serve to reduce antigen presentation and T cell killing of infected targets. These viral mechanisms occur posttranslationally (22, 23) and should not affect the contribution of HLA class I signal peptides to enhancing HLA-E expression. This in turn may serve to allow continued evasion of NK cell responses through enhanced NKG2A inhibition among those individuals with HLA haplotypes that provide ample signal peptide to bind HLA-E. HIV encodes a peptide (AISPRTLNA, AA9) that may further exploit the inhibitory effects of HLA-E, but discrepancies regarding the effects of this peptide on HLA-E expression, NKG2A binding, and NK cell killing have been reported (24, 25). NKG2A-expressing CD8 T cells are involved in antiviral responses (26), but the functional assays that we used are not appropriate for evaluating CD8⁺ T cell responses, and thus, we cannot rule out a role for CD8 T cells in the genetic data presented herein. Although NKG2C, an activating receptor that also binds HLA-E (27), may play some role in the pathway that we delineate, signaling through NKG2A dominates and overrides NKG2C signaling (28).

These data show that expression level variation participates in the complex patterns of HLA associations in HIV disease, a pattern recognized for class I in other species (29). Blockade of HLA-E:NKG2A-mediated inhibition in vivo is a therapeutic strategy being explored through clinical trials of an antibody against NKG2A (monalizumab) for treatment of rheumatoid arthritis (NCT02331875), cancer (NCT 02557516, NCT02643550, NCT02459301, NCT02671435), and stem-cell transplantation (NCT02921685), because a role for HLA-E-mediated immunosuppression is recognized in these disorders (30, 31). Our data suggest that antagonizing HLA-E/NKG2A interactions, perhaps in combination with other therapies, may provide benefit in HIV disease. This might be an attractive approach in HIV cure strategies. Genetic validation of NKG2A as a therapeutic target in additional diseases by testing for effects of HLA-A and HLA-B -21 genotypes may rationalize the use of anti-NKG2A therapy in other disorders.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/86/suppl/DC1 Materials and Methods Table S1 to S3 Figs. S1 to S3 References (32-49)

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CANCER IMMUNOTHERAPY

Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors

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Immune checkpoint inhibitors (ICIs) targeting the PD-1/PD-L1 axis induce sustained clinical responses in a sizable minority of cancer patients. We found that primary resistance to ICIs can be attributed to abnormal gut microbiome composition. Antibiotics inhibited the clinical benefit of ICIs in patients with advanced cancer. Fecal microbiota transplantation (FMT) from cancer patients who responded to ICIs into germ-free or antibiotic-treated mice ameliorated the antitumor effects of PD-1 blockade, whereas FMT from nonresponding patients failed to do so. Metagenomics of patient stool samples at diagnosis revealed correlations between clinical responses to ICIs and the relative abundance of Akkermansia muciniphila. Oral supplementation with A. muciniphila after FMT with nonresponder feces restored the efficacy of PD-1 blockade in an interleukin-12dependent manner by increasing the recruitment of CCR9*CXCR3*CD4* T lymphocytes into mouse tumor beds.

ancer immunotherapy has become highly successful against an array of distinct hematological and solid metastatic malignancies (1-6). Administration of immune checkpoint inhibitors (ICIs) unleashes T lymphocytemediated immune responses by suppressing the interaction of T cell inhibitory receptors with their cognate ligands on tumor or stromal cells (7). The most widely used ICIs are monoclonal antibodies (mAbs) targeting programmed cell death protein 1 (PD-1) and its ligand PD-L1 (7). PD-1 blockade is highly efficacious against advanced melanoma, non-small cell lung cancer (NSCLC), and renal cell carcinoma (RCC). Primary resistance, observed in 60 to 70% of cases (3, 5, 8), has been attributed to low mutational burden, poor intrinsic antigenicity of tumor cells (9, 10), absence of priming by potentially immunogenic pretreatment with chemo- or radiotherapy (II), defective antigen presentation during the priming phase (12), local immunosuppression by extracellular metabolites (13), and functional exhaustion of tumor-infiltrating lymphocytes (13-15).

Recent work in mice has highlighted the key role of the gut microbiota in mediating tumor responses to chemotherapeutic agents and im-

munotherapies targeting PD-L1 or cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (16-21). Therefore, we explored the possibility that dysbiosis associated with malignant disease or concomitant antibiotic (ATB) use could influence primary resistance to PD-1 blockade in tumorbearing mice and cancer patients.

Initially, we compared the therapeutic efficacy of PD-1 mAb alone or combined with CTLA-4 mAb in mice with established MCA-205 sarcoma and RET melanoma. Mice were reared in specific pathogen-free (SPF) conditions and treated for 14 days with broad-spectrum combination ATB (ampicillin + colistin + streptomycin) or left untreated. ATB treatment significantly compromised the antitumor effects and survival of mice treated with PD-1 mAb alone or in combination with CTLA-4 mAb (Fig. 1, A and B).

We next addressed the impact of ATB on patients with advanced NSCLC (n = 140), RCC (n = 140) 67), or urothelial carcinoma (n = 42) who received PD-1/PD-L1 mAb after one or several prior therapies. Out of all 249 patients, 69 (28%) were prescribed ATB (β -lactam^{+/-} inhibitors, fluoroquinolones, or macrolides) within 2 months before, or 1 month after, the first administration of PD-1/PD-L1 mAb. Patients generally took ATB orally for common indications (dental, urinary, and pulmonary infections). There were no major statistical differences in baseline clinical characteristics between ATB-treated and untreated patients (tables S1 to S6). Progression-free survival (PFS) and overall survival (OS) were significantly shorter in the ATB-treated group when all patients were combined (Fig. 1C). Similarly, PFS and/or OS were shorter in ATB-treated groups when individual tumor types were considered (Fig. 1, D and E, and fig. S1, A to C). In univariate and multivariate Cox regression analyses, ATB represented a predictor of resistance to PD-1 blockade. independent from classical prognostic markers in NSCLC and RCC (tables S7 to S9). A validation cohort of 239 advanced NSCLC patients confirmed the negative impact of ATB uptake on OS during PD-1/PD-L1 inhibition (fig. S1D). In contrast, proton pump inhibitors, a medication that can also alter the microbiota composition. failed to affect PFS or OS in these patients (fig. S2) (22). On the basis of previous observations that ATB can transiently change the composition of the gut microbiome (23), we hypothesized that dysbiosis might affect the therapeutic

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To explore the composition of the gut microbiota, we used quantitative metagenomics by shotgun sequencing, reaching >20 million short DNA sequence reads per sample, followed by analysis of the results in a reference catalog of 9.9 million genes (24). Total DNA was extracted from 100 patients diagnosed with NSCLC (n =60) and RCC (n = 40) before starting therapy and serially after PD-1 blockade (tables S10 to S13). The higher richness of the samples evaluated at the gene count or metagenomic species (MGS) levels correlated with the clinical response defined by the absence of progression of disease, 6 months after initiation of ICIs based on RECIST 1.1 criteria (Fig. 2A) (25). Stool richness increased at the MGS level over the course of therapy, more in RCC patients than in NSCLC patients (fig. S3). For each sample, MGS occurrence was visualized using "barcodes" (i.e., heat maps reflecting the abundance of 50 marker genes for each MGS) (fig. S4). The taxonomical annotation of each MGS was based on gene homology to previously sequenced organisms (using blastN against the nucleotide and whole-genome shotgun data banks).

When segregating responders (R) from nonresponders (NR) (according to the best clinical response as assessed by RECIST1.1), we observed an overrepresentation of unclassified and classified Firmicutes, as well as distinct bacterial genera (such as Akkermansia and Alistipes) (Fig. 2B and fig. S4). The commensal that was most significantly associated with favorable clinical outcome in both NSCLC and RCC was A. muciniphila (P = 0.004 considering all patients, P = 0.003)excluding ATB-treated patients) (Fig. 2B; fig. S4, A and B; and tables S10 to S13). When analyzing PFS according to RECIST 1.1, A. muciniphila was also enriched in patients with PFS longer than 3 months relative to those with PFS shorter than 3 months, both in the whole cohort (P = 0.028, fig. S5A) and when excluding patients on ATB (P = 0.007; Fig. 2C and fig. S5B). A. muciniphila was also enriched when analyzing the NSCLC cohort alone (P = 0.045 with or without ATB, fig. S6A; P = 0.026 excluding ATB, fig. S6B) along with other commensals such as Ruminococcus spp., Alistipes spp., and Eubacterium spp., with a relative underrepresentation of Bifidobacterium adolescentis, B. longum, and Parabacteroides distasonis. More precisely, the fecal presence of A. muciniphila was detectable in 69% (11/16) and 58% (23/40) of patients exhibiting a partial response or stable disease, respectively, whereas it could only be detected in 34% (15/44) of patients who progressed or died (P = 0.007, Fig. 2D). A validation cohort of 53 patients (27 NSCLC and 26 RCC) confirmed that A. muciniphila was enriched in patients with the best clinical response and PFS longer than 3 months (fig. S7 and table S14). These findings show that A. muciniphila was overrepresented at diagnosis in the feces of patients who later benefited from PD-1 inhibition.

In an attempt to link gut microbial content to systemic immune tone, we analyzed memory T cell responses from peripheral blood, elicited against microbiota after the initiation of PD-1 blockade. The recall response of circulating

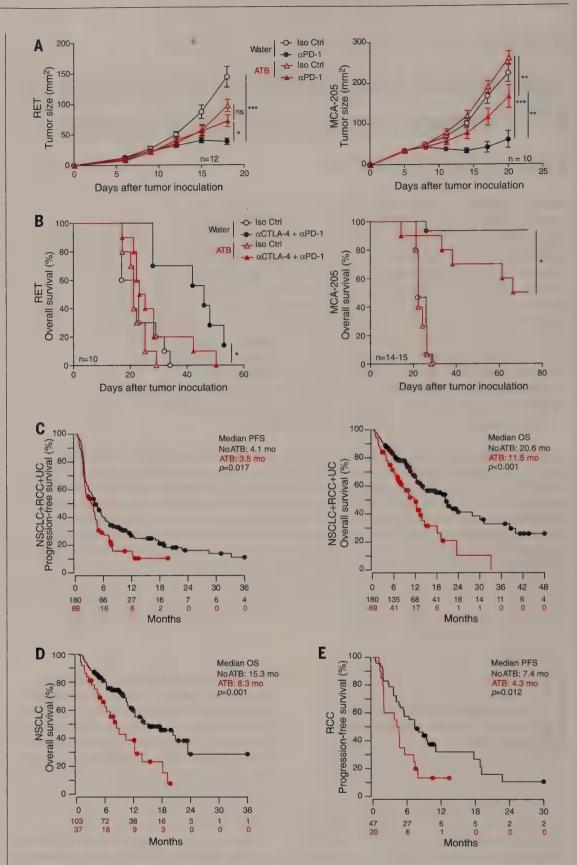


Fig. 1. Antibiotics compromise the efficacy of PD-1 blockade in mouse tumor models and cancer patients. (A) Tumor growth kinetics of RET melanoma (left) and MCA-205 sarcoma in mice (right) treated with four injections of PD-1 (clone RMP1-14) mAb (α PD-1) or isotype control mAb (Iso Ctrl) in the presence or absence of broad-spectrum antibiotics (ATB). Data are means ± SEM of tumor sizes for 10 to 12 mice per group. (**B**) Survival curves of RET-bearing mice (left) and MCA-205—bearing mice (right) treated with PD-1 mAb combined with CTLA-4 mAbs. Each line represents one survival curve for each group of five mice from two or three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.01 [analysis of variance (ANOVA) and log-rank (Mantel-Cox) analysis]; ns, not significant. (**C** to **E**) Kaplan-Meier estimates for progression-free survival (PFS) or overall survival (OS) of cancer patients. (C) All cancer patients (n = 249); (D) patients with advanced non-small cell lung cancer (NSCLC, n = 140, fig. S1A; fig. S1D for the validation cohort); (E) renal cell carcinoma (RCC, n = 67, fig. S1B). [See fig. S1C and tables S1 to S6 for data on patients with urothelial carcinoma (n = 42) treated with PD-1/PD-L1 mAb who did or did not receive ATB.] The points represent data censored at the last time the patient was known to be alive and without progression. P values are shown [log-rank (Mantel-Cox) analysis].

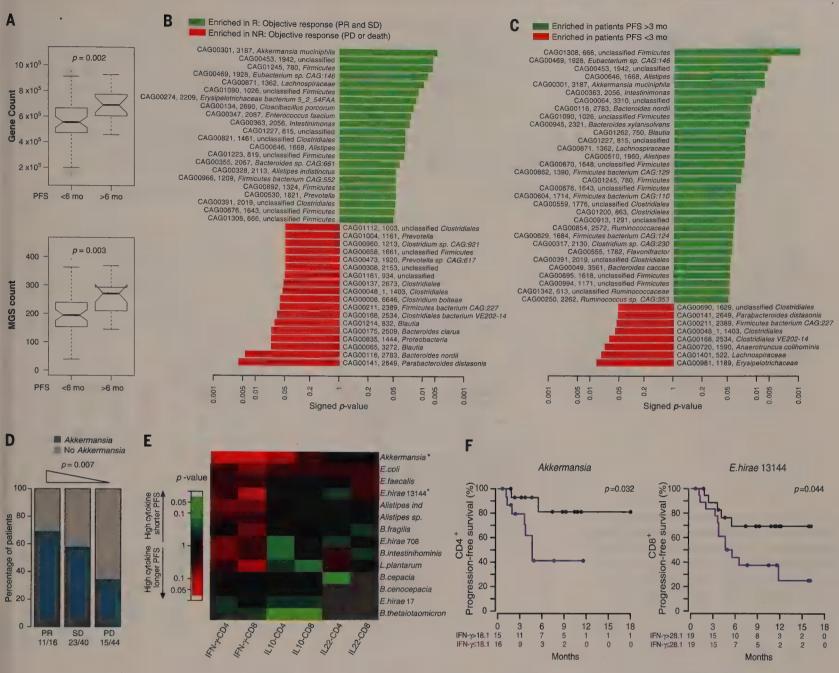
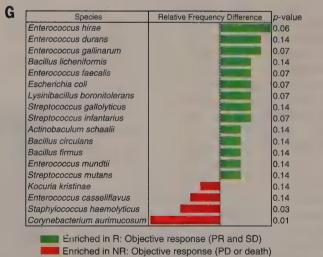
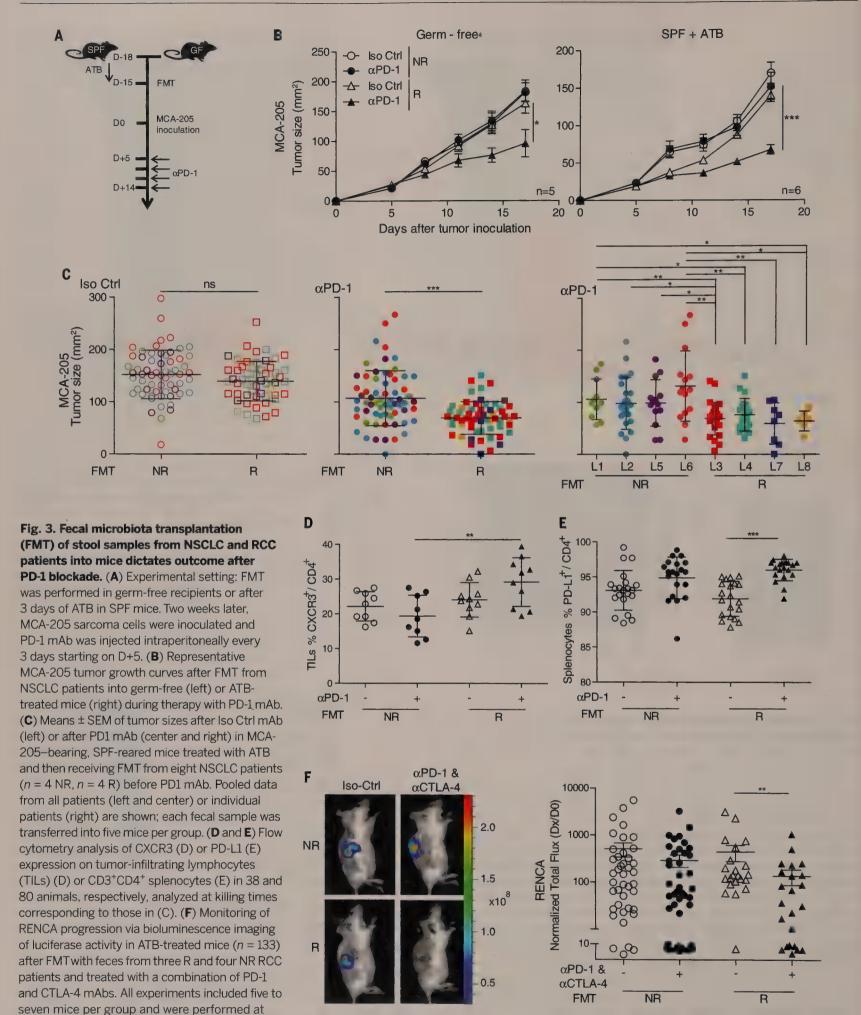


Fig. 2. Metagenomic analysis of fecal samples predicts response at **3 months of PD-1 mAb treatment in cancer patients. (A)** Shotgun sequencing of fecal samples at diagnosis with representation of gene and MGS counts for all cancer patients according to clinical outcome (PFS at 6 months). Data are means ± SEM of counts for patients experiencing PFS shorter or longer than 6 months. Gene or MGS richness did not predict PFS at 3 months. (B and C) Shotgun sequencing of fecal samples at diagnosis with representation of the relative abundance of each MGS in responders (R) (partial response or stable disease) over nonresponders (NR) (progression or death) defined using the best clinical response according to RECIST1.1 criteria (B) or PFS at 3 months (C) and the corresponding P value on the entire cohort of n = 100 (60 NSCLC and 40 RCC) patients (B) and excluding those who took ATB (C) (fig. S5B), n = 78 (42 R, 36 NR); see also fig. S5A for all patients. TO samples were analyzed; when not available, T1 specimens were used, as there was no statistical difference between TO and T1 (fig. S3A). (D) Frequency of patients with detectable A. muciniphila in their feces according to PR (partial response), SD (stable disease), or PD (progressive disease) clinical status, as assessed by metagenomics and analyzed by Cochran-Armitage test. (E and F) Circulating memory Tcell immune responses directed against commensals detected during PD-1 blockade and evaluation of the time to progression. (E) Heat map of the P values for each cytokine and each commensal, segregating NSCLC+RCC patients' PFS according to the median value of cytokine production of the whole cohort. Significant P values (<0.05, Student t test) are indicated with an asterisk for the



relevant commensals. (F) Univariate analysis and Kaplan-Meier curves showing PFS for peripheral blood memory Th1 and Tc1 immune responses directed against A. muciniphila and E. hirae 13144, respectively. See fig. S9 for PFS curves for nonspecific T cell receptor-driven cytokine release. (G) Culturomics-based analyses of fecal samples in 16 R and 16 NR NSCLC patients (defined as the best clinical outcome) before therapy, each commensal colony having been identified by mass spectrometry. Colored bars show relative frequencies of each commensal in all fecal cultures in R over NR patients, with P values of the difference shown at the right.



least twice in similar conditions yielding similar results. Data are means \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA and Student t test).

Dx, last IVIS (bioluminescent imaging) measurement; D0, day of randomization.

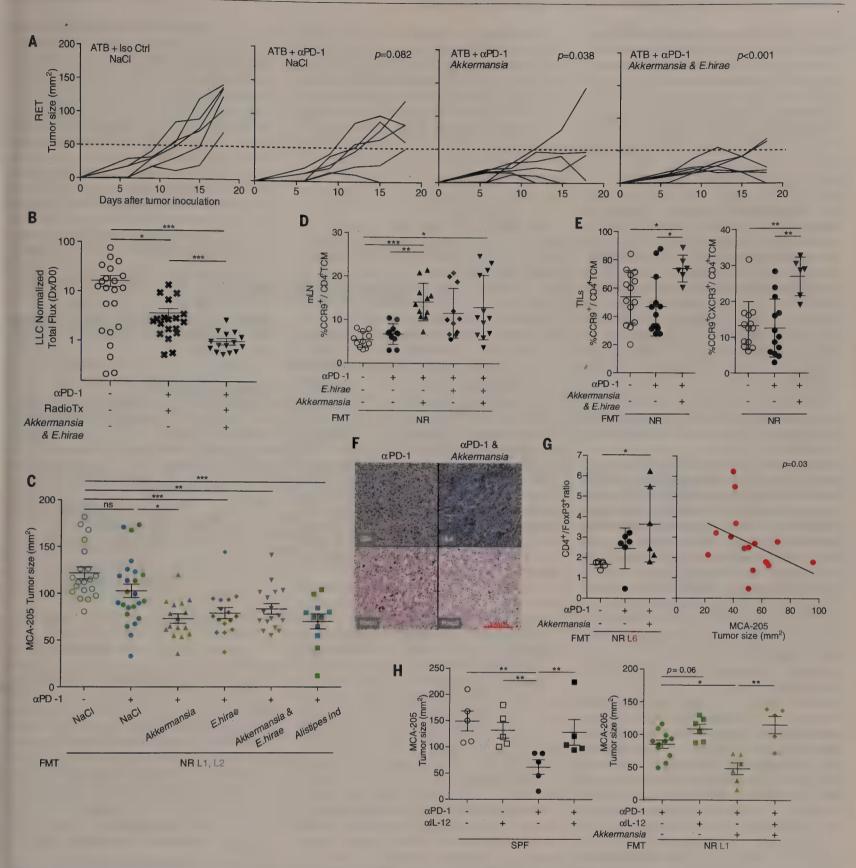


Fig. 4. Biological importance of *A. muciniphila* **during anticancer PD-1 blockade treatment.** (**A** and **B**) ATB-induced dysbiosis was restored by oral administration of *A. muciniphila* (*Akkermansia*) alone or combined with *Enterococcus hirae* 13144 (*E. hirae*) to recipient mice receiving PD-1 mAb. Commensals and PD-1 mAb regimens were administered five times every 3 days against RET melanomas (A) and four times in luciferase-expressing orthotopic LLC non—small cell lung cancers treated, or not, with radiotherapy plus PD-1 mAb (B). (**C**) Means ± SEM of tumor sizes at time of killing in mice exhibiting NR FMT-induced dysbiosis and compensated with *A. muciniphila* alone or combined with *E. hirae* (also refer to fig. S12) or control bacteria (fig. S13) during PD-1 mAb—based therapy. Each color represents one NR donor of feces transferred into five mice per group. (**D** and **E**) Flow cytometry analysis of CCR9 and CXCR3 expression in mLN-residing CD4⁺ TCM at 48 hours (D) and at D+17 among the tumor-infiltrating lymphocytes (TILs) (E) after the first

injection of PD-1 mAb in ATB-treated animals compensated with a mixture of A. muciniphila and E. hirae. Data are pooled from two independent experiments composed of five to seven mice per group; each symbol represents one mouse. (**F** and **G**) Immunohistochemical determination of CD4 and FoxP3 infiltrates in D+10-treated tumor beds [representative micrograph in (F)] calculated by image analyzer for the experimental setting described in Fig. 3C and calculation of the ratios between these two values on the whole tumor sample [(G), left]; Spearman correlation between CD4/FoxP3 ratios and tumor size at the time of killing [(G), right]. (**H**) Effects of neutralizing IL-12p40 mAb on the anticancer efficacy of PD-1 inhibition alone (left) or combined with Akkermansia (right). Data are means \pm SEM of tumor sizes at killing; each symbol represents one tumor and each group comprises five mice. One representative experiment out of four is shown. *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA and Student t test). Dx, last IVIS measurement; D0, day of randomization.

CD4⁺ and CD8⁺ T cells harvested from 27 NSCLC and 28 RCC patients under PD-1 blockade was measured upon coculture with autologous monocytes preincubated with distinct commensals (Fig. 2E and tables S15 and S16). In this setting, the secretion of cytokines by major histocompatibility complex class II-restricted CD4⁺ T cells (Th1, Th22, or Tr1) was quantified (figs. S8 and S9). The only immune responses that correlated with the clinical outcome during PD-1 mAbtargeted ICI were the Th1 (Fig. 2F) and Tc1 (fig. S10A) cell reactivity against A. muciniphila, interferon-γ (IFN-γ) release above the median being associated with prolonged PFS. In contrast, no association was found between clinical outcome and IFN-y, interleukin-10 (IL-10), or IL-22 secretion stimulated by nonspecific cross-linking of the T cell receptor (fig. S10B) or memory T cell responses to 10 other commensals with the exception of Enterococcus hirae 13144 (E. hirae) for the Tc1 response (Fig. 2F), as previously reported (19). We also observed a higher incidence of cultivatable E. hirae in R NSCLC patients than in NR NSCLC patients among 32 stool samples tested at diagnosis (Fig. 2G), further supporting the relevance of E. hirae to predict best clinical outcome.

To establish a cause-effect relationship between the anticancer efficacy of PD-1 blockade and the dominance of distinct commensal species in clinical responses, we recolonized ATB-treated mice reared in SPF conditions (or, alternatively, germ-free animals) by fecal microbiota transplantation (FMT) using patient stool. Such "avatar mice" were prepared by oral gavage of feces harvested at diagnosis from eight different NSCLC patients, four R and four NR (defined using best clinical outcome). Two weeks later, C57BL/6 avatar mice were inoculated with MCA-205 tumor cells, then treated 5 days later with PD-1 mAb (Fig. 3A). Stool samples from clinical R conferred sensitivity, whereas those from NR patients conveyed resistance to PD-1 blockade with a similar efficiency in ATB-treated or germ-free avatar mice (Fig. 3B and table S17). Although stool composition failed to influence the natural progression of MCA-205 sarcomas, it did so after PD-1 blockade (Fig. 3C). FMT from R patients (but not from NR patients) into avatar mice caused tumor growth delay (Fig. 3C, center and right), accumulation of CXCR3⁺CD4⁺ T cells in the tumor microenvironment (Fig. 3D), and up-regulation of PD-L1 in splenic T cells (Fig. 3E) after PD-1 blockade. These findings could be corroborated by FMT from seven RCC patients into ATB-treated BALB/c mice that were then orthotopically implanted with luciferase-expressing renal cancer (RENCA) cells resistant to PD-1 monotherapy and treated with a combination of CTLA-4 and PD-1 mAbs (fig. S11 and table S18). Bioluminescence monitoring revealed that stool samples from R patients restored the antitumor activity of simultaneous CTLA-4 and PD-1 blockade, unlike those from NR patients (Fig. 3F). Together, these results suggest that the intestinal microbiota from patients influences the outcome of ICIs in avatar mice.

To validate the biological importance of the microbiota identified in the metagenomic analysis of patients with a favorable clinical outcome, we colonized intestines from mice with A. muciniphila alone or combined with other commensals in several conditions of gut dysbiosis. First, ATBmediated sterilization of the microbiome was followed by natural recolonization supplemented with five oral gavages of A. muciniphila, alone or together with the goblet cell-stimulating E. hirae (17, 19), in an attempt to restore responsiveness to PD-1 blockade in RET melanoma-bearing hosts reared in SPF conditions. A. muciniphila alone or combined with E. hirae reinstated the anticancer effects of PD-1 blockade that were previously inhibited by ATB (Fig. 1A, Fig. 4A, and fig. S12A). Second, we used an orthotopic luciferaseexpressing Lewis lung carcinoma (LLC) model amenable to combination treatment by local radiotherapy and PD-1 blockade (26). Again, oral gavages with A. muciniphila and E. hirae increased the efficacy of PD-1 blockade with respect to tumor growth, as measured by whole-body imaging (Fig. 4B). Finally, we confirmed that monocolonization with A. muciniphila or bicolonization of A. muciniphila plus E. hirae reversed the compromised efficacy of PD-1 blockade observed after the recolonization of germ-free or ATB-treated SPF mice with FMT from NR patients (Fig. 4C and fig. S12B). In addition to this, Alistipes indistinctus, which was found to be overrepresented among NSCLC responders, was efficient in restoring the efficacy of this ICI in avatar mice (Fig. 4C and fig. S12); other unrelated commensals failed to do so (fig. S13).

Next, we analyzed the immunological changes elicited by oral gavage with a combination of A. muciniphila and E. hirae in mesenteric lymph nodes (mLN), tumor draining LN (dLN), and tumor beds. Central memory (TCM) CD4⁺ T cells expressing the small intestine-associated chemokine receptor CCR9 and/or the Th1-associated chemokine receptor CXCR3 accumulated 48 hours after the first injection in mLN (Fig. 4D) and were observed in dLN (fig. S14A) and tumor beds upon killing (Fig. 4E and fig. S14, B and C). Immunohistochemical studies revealed the formation of intratumoral granulomas (fig. S15) and increased CD4/Foxp3 ratios in tumors from animals cotreated with PD-1 mAb and A. muciniphila (Fig. 4, F and G). Moreover, A. muciniphila and E. hirae (19) induced dendritic cells to secrete IL-12 (fig. S16A), a Th1 cytokine involved in the immunogenicity of PD-1 blockade in eubiotic conditions (Fig. 4H, left, and fig. S16B) as well as in the adjuvant effects of A. muciniphila in dysbiotic settings (Fig. 4H, right), as previously shown for B. fragilis in the context of CTLA-4 blockade (20).

Our findings show that the gut microbiome markedly influences the outcome of PD-1 blockade in mice and patients. Several issues remain unresolved. The mechanisms accounting for the immunomodulatory effects of *A. muciniphila*, one of the most abundant bacteria in the ileum microbiota, remain unclear (27). Cancer patients face stresses that can cause gut barrier dysfunction and systemic endotoxemia. By reinforcing

intestinal barrier integrity and reducing systemic inflammation, A. muciniphila, Clostridiales, and Ruminococcaceae might generate "homeostatic" consortia of commensals that prevent leaky colon and systemic immunosuppression (28-30). In parallel, unleashing T cells by PD-1 blockade alleviated small intestine tolerance, thereby eliciting local and systemic recall Th1 immune responses against A. muciniphila that might improve cancer immunosurveillance. Finally, a comprehensive analysis of stool composition after a chronic shift of the microbiome enforced by commensals endowed with anticancer effects might unveil further functional links between the microbial ecosystem and anticancer immunosurveillance (28). Irrespective of these remaining questions, our findings suggest that the microbiome governs the cancerimmune set point of cancer-bearing individuals (30) and that manipulating the gut ecosystem to circumvent primary resistance to ICIs may be-

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/91/suppl/DC1 Materials and Methods Figs. S1 to S16 Tables S1 to S18 References (31–35)

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CANCER IMMUNOTHERAPY

Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients

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Preclinical mouse models suggest that the gut microbiome modulates tumor response to checkpoint blockade immunotherapy; however, this has not been well-characterized in human cancer patients. Here we examined the oral and gut microbiome of melanoma patients undergoing anti-programmed cell death 1 protein (PD-1) immunotherapy (n=112). Significant differences were observed in the diversity and composition of the patient gut microbiome of responders versus nonresponders. Analysis of patient fecal microbiome samples (n=43, 30 responders, 13 nonresponders) showed significantly higher alpha diversity (P<0.01) and relative abundance of bacteria of the Ruminococcaceae family (P<0.01) in responding patients. Metagenomic studies revealed functional differences in gut bacteria in responders, including enrichment of anabolic pathways. Immune profiling suggested enhanced systemic and antitumor immunity in responding patients with a favorable gut microbiome as well as in germ-free mice receiving fecal transplants from responding patients. Together, these data have important implications for the treatment of melanoma patients with immune checkpoint inhibitors.

remendous advances have been made in the treatment of melanoma and other cancers by using immune checkpoint inhibitors targeting the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1); however, responses to these therapies are often heterogeneous and not durable (1–3). It has recently emerged that factors beyond tumor genomics influence cancer development and therapeutic responses (4–7), including host factors such as the gastrointestinal (gut) microbiome (8–10). A number of studies have shown that the gut microbiome may influence antitumor immune responses by means of innate

and adaptive immunity (11, 12) and that therapeutic responses may be improved through its modulation (13, 14); however, this has not been extensively studied in cancer patients.

To better understand the role of the microbiome in response to immune checkpoint blockade, we prospectively collected microbiome samples from patients with metastatic melanoma starting treatment with anti-PD-1 therapy (n=112 patients) (fig. S1 and table S1). Oral (buccal) and gut (fecal) microbiome samples were collected at treatment initiation, and tumor biopsies and blood samples were collected at matched pretreatment time points when possible, to assess for genomic alterations

as well as the density and phenotype of tumorinfiltrating and circulating immune cell subsets (Fig. 1A and fig. S2). Taxonomic profiling using 16S ribosomal RNA (rRNA) gene sequencing was performed on all available oral and gut samples, with metagenomic whole-genome shotgun (WGS) sequencing performed on a subset (n = 25). Eligible patients (n = 89) were classified as responders (R, n = 54) or nonresponders (NR, n = 35) on the basis of radiographic assessment using the response evaluation criteria in solid tumors (RECIST 1.1) (15) at 6 months after treatment initiation. Patients were classified as R if they achieved an objective response (complete or partial response or stable disease lasting at least 6 months) or NR (progressive disease or stable disease lasting less than 6 months). This classification accounts for the subset of patients who may derive long-term disease benefit despite not achieving a bona fide RECIST response and has been used in numerous published studies of patients on checkpoint blockade (16-19). Of note, patients in R and NR groups were similar with respect to age, gender, primary type, prior therapy, concurrent systemic therapy, and serum lactate dehydrogenase (table S2). Prior genomic analyses have demonstrated that patients with tumors that have a higher mutational load are more likely to respond to anti-CTLA-4 (16, 20, 21) or anti-PD-1 therapy (21-24); however, a high mutational load alone appears neither sufficient nor essential for response. In this cohort, the total number of mutations and specific melanoma driver mutations were within comparable parameters between R and NR after anti-PD-1 therapy (fig. S3), though the number of tumors available for sequencing (n = 10, R = 7,NR = 3) was limited and may have reduced our ability to detect a significant association between mutational burden and response.

We first assessed the landscape of the oral and gut microbiome in all available samples in patients (n = 112) with metastatic melanoma using 16S sequencing, noting that both communities were relatively diverse with a high abundance of bacteria of the order Lactobacillales in the oral microbiome and Bacteroidales in the fecal microbiome (Fig. 1B). Bipartite network analysis (25) demonstrated a clear separation of community structure between the oral and fecal microbiomes in terms of both matched and aggregate samples (fig. S4). suggesting that these communities are distinct in terms of their compositional structure. Loss of microbial diversity (dysbiosis) is associated with chronic health conditions (26-28) and cancer (8-10)and is also associated with poor outcomes of certain forms of cancer therapy, including allogeneic stem

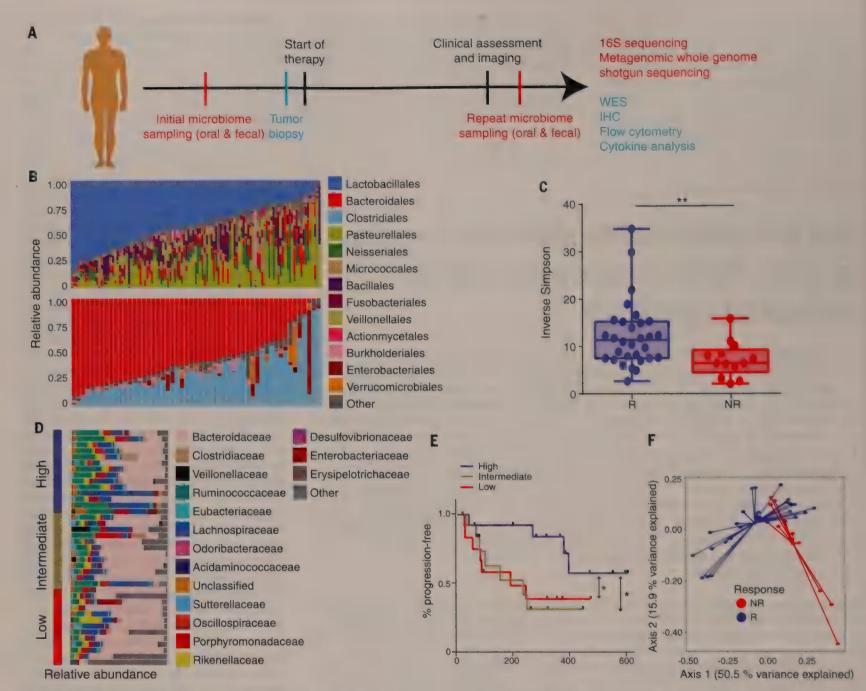


Fig. 1. Higher gut microbiome diversity is associated with improved response to anti-PD-1 immunotherapy in patients with metastatic melanoma. (A) Schema of sample collection and analyses. (B) Stacked bar plot of phylogenetic composition of common bacterial taxa (>0.1% abundance) at the order level in oral (n = 109, top) and fecal (n = 53, bottom)samples by 16S rRNA sequencing. (C) Inverse Simpson diversity scores of the gut microbiome in R (n = 30) and NR (n = 13) to anti-PD-1 immunotherapy by Mann-Whitney U rank sum (MW) test. Error bars represent the distribution of diversity scores. (**D**) Phylogenetic composition of fecal samples (n = 39)

at the family level (>0.1% abundance) at baseline. High [blue, >11.63 (inverse Simpson score), n = 13], intermediate (gold, 7.46 to 11.63, n = 13), and low (red, <7.46, n = 13) diversity groups were determined using tertiles of inverse Simpson scores. (E) Kaplan-Meier (KM) plot of PFS by fecal diversity: high (median PFS undefined), intermediate (median PFS = 232 days), and low (median PFS = 188 days). High versus intermediate diversity (HR 3.60, 95% Cl 1.02 to 12.74) and high versus low (HR 3.57, 95% Cl 1.02 to 12.52) by univariate Cox model. (F) Principal coordinate analysis of fecal samples (n = 43) by response using weighted UniFrac distances. *P < 0.05; **P < 0.01.

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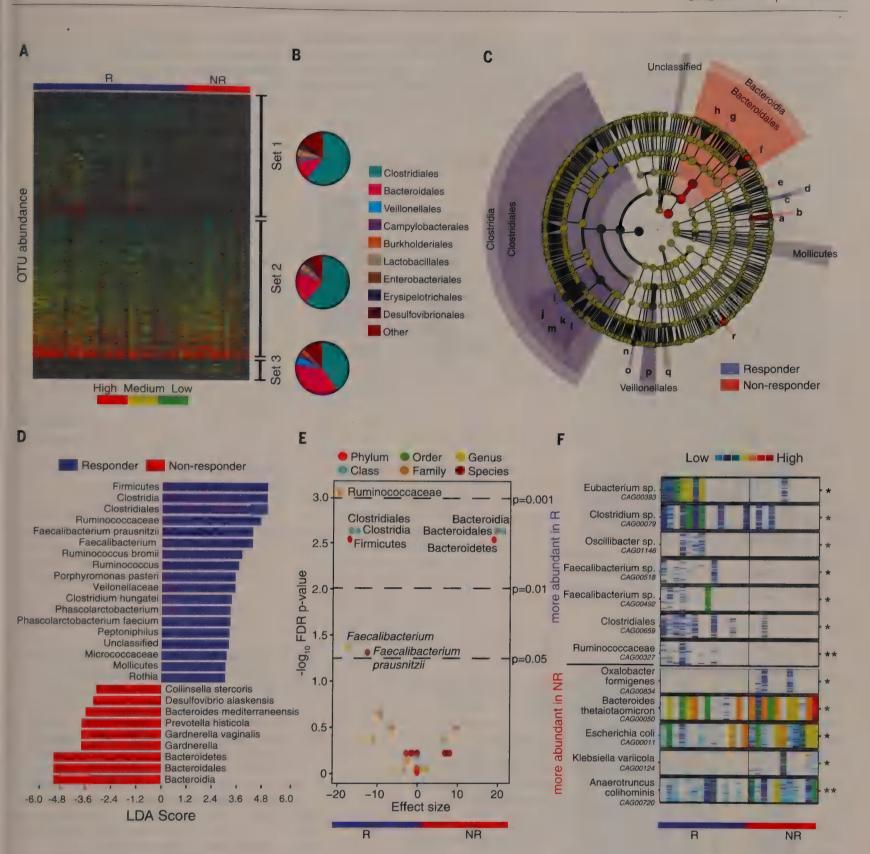
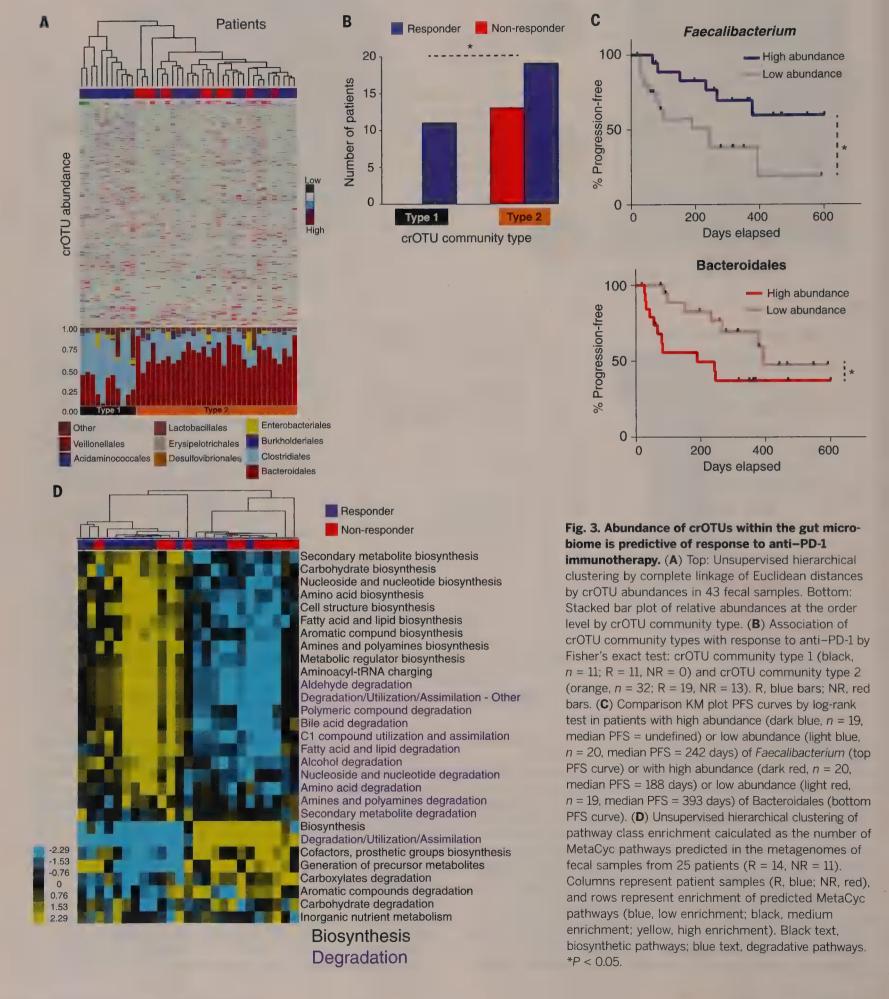


Fig. 2. Compositional differences in the gut microbiome are associated with responses to anti–PD-1 immunotherapy. (A) Heatmap of OTU abundances in R (n=30) and NR (n=13). Columns denote patients grouped by response and sorted by diversity within R and NR groups; rows denote bacterial OTUs grouped into three sets according to their enrichment and/or depletion in R versus NR and then sorted by mean abundance within each set. Set 1 (enriched in R), Set 2 (unenriched), and Set 3 (enriched in NR). (B) Phylogenetic composition of OTUs within each set described in (A) at the order level. (C) Taxonomic cladogram from LEfSe showing differences in fecal taxa. Dot size is proportional to the abundance of the taxon. Letters correspond to the following taxa: (a) *Gardnerella vaginalis*, (b) Gardnerella, (c) *Rothia*, (d) *Micrococcaceae*, (e) *Collinsella stercoris*, (f) *Bacteroides mediterraneensis*, (g) *Porphyromonas pasteri*, (h) *Prevotella histicola*, (i) *Faecalibacterium prausnitzii*, (j) *Faecalibacterium*, (k) *Clostridium hungatei*, (l) *Ruminococcus bromii*, (m) Ruminococcaceae, (n) *Phascolarctobacterium*

faecium, (o) Phascolarctobacterium, (p) Veilonellaceae, (q) Peptoniphilus, and (r) Desulfovibrio alaskensis. (**D**) Linear discriminant analysis (LDA) scores computed for differentially abundant taxa in the fecal microbiomes of R (blue) and NR (red). Length indicates effect size associated with a taxon. P = 0.05 for the Kruskal-Wallis H statistic; LDA score > 3. (**E**) Differentially abundant gut bacteria in R (blue) versus NR (red) by MW test [false-discovery rate (FDR)-adjusted] within all taxonomic levels. (**F**) Pairwise comparisons by MW test of abundances of metagenomic species identified by metagenomic WGS sequencing in fecal samples (n = 25) for R (n = 14, blue) and NR (n = 11, red). *P < 0.05; **P < 0.01. Colors reflect gene abundances visualized as "barcodes" with the following order of intensity: white (0) < light blue < blue < green < yellow < orange < red for increasing abundance, where each color change corresponds to a fourfold abundance change. In these barcodes, metagenomic species appear as vertical lines (coabundant genes in a sample) colored according to the gene abundance.

cell transplant (29). Based on these data, we examined the diversity of the oral and gut microbiomes in eligible patients on anti-PD-1 therapy and found that alpha diversity, or within-sample diversity, of the gut microbiome was significantly

higher in R (n = 30) compared to NR (n = 13)using several indices (P < 0.01; Fig. 1C and fig. S5). No significant differences were observed in the oral microbiome (R = 54, NR = 32, P = 0.11; fig. S6). We then tested the relationship of diversity and progression-free survival (PFS) in our cohort by stratifying patients based on tertiles of inverse Simpson scores, demonstrating that patients in the highest tertile of fecal alpha diversity had significantly prolonged PFS compared to those with



intermediate or low diversity (P = 0.02 and 0.04, respectively; Fig. 1, D and E, and fig. S7). No differences in PFS were noted when comparing diversity of the oral microbiome (fig. S8). Importantly, upon visualizing beta diversity, or betweensample diversity, with weighted UniFrac distances (30) by principal coordinate analysis, we found a notable clustering effect by response status in the gut microbiome of these patients, which was not observed in the oral microbiome (Fig. 1F and fig. S8E).

Because compositional differences in the microbiome may also influence cancer development and response to therapy (12, 14, 15, 23), we sought to determine if differences existed in the oral or gut microbiomes of R and NR to anti-PD-1 therapy. To test this, we first compared an enrichment of operational taxonomic units (OTUs) in R versus NR, demonstrating that distinct sets of rare low abundance OTUs were associated with response to anti-PD-1 therapy, with enrichment of orders Clostridiales in R and Bacteroidales in NR in the gut microbiome (P < 0.01; Fig. 2, A and B, and fig. S9, A and C). No significant differences in enrichment were noted in the oral microbiome of R versus NR (fig. S9, B and D, and fig. S10). To further explore these findings, we performed high-dimensional class comparisons using linear discriminant analysis of effect size (LEfSe) (31), which again demonstrated differentially abundant bacteria in the fecal microbiome of R versus NR in response to anti-PD-1 therapy, with Clostridiales order and Ruminococcaceae family enriched in R and Bacteroidales order enriched in NR (Fig. 2, C and D). No major differences were observed in the oral microbiome between R and NR, with the exception of higher Bacteroidales in NR in response to anti-PD-1 therapy (fig. S11). Pairwise comparisons were then performed for bacterial taxa at all levels by response. In addition to confirming the previous taxonomic differences, these analyses identified the Faecalibacterium genus as significantly enriched in R (Fig. 2E and table S3). Metagenomic WGS sequencing further confirmed enrichment of Faecalibacterium species in addition to others in R, whereas Bacteroides thetaiotaomicron, Escherichia coli, and Anaerotruncus colihominis were enriched in NR (Fig. 2F and table S4). Importantly, the gut microbiome was shown to be relatively stable over time in a limited number of longitudinal samples tested (fig. S12).

We next asked whether bacterial composition and abundances within the gut and/or oral microbiomes of patients were associated with a specific treatment outcome to anti-PD-1 therapy. We grouped all identified OTUs into clusters of related OTUs (crOTUs) by means of construction of a phylogenetic tree from sequence-alignment data (32). This technique involves comparison of abundances of different potential groupings of bacteria based on 16S sequence similarity and helps address the sparse distribution of OTU abundances observed in the absence of this approach (fig. S13). Unsupervised hierarchical clustering of crOTU abundances within the gut and oral microbiomes was then performed without input of response data. We found that patients segregated

into two distinct community types. Type 1 was composed entirely of R and was enriched for Clostridiales, whereas type 2 comprised a mixture of R and NR (P = 0.02) and was enriched for Bacteroidales (Fig. 3A). To better understand compositional differences between these crOTU community types, we again performed pairwise comparisons of the gut microbiota and identified a pattern very similar to that seen when clustering by response, with Clostridiales and Ruminococcaceae enriched in type 1, and Bacteroidales enriched in type 2 (fig. S14A and table S5). Further, these communities clustered distinctly using principal coordinate analysis of weighted UniFrac distances (fig. S14B). Analysis of crOTUs in the oral microbiome revealed no apparent relationship to treatment response (fig. S15, A and B).

To explore how specific bacterial taxa affect patient treatment response, we compared PFS following anti-PD-1 therapy as it related to the "top hits" that were consistently observed across our analyses. From the Ruminococcaceae family of the Clostridiales order, we focused on the Faecalibacterium genus in R and Bacteroidales order in NR and stratified patients into high versus low categories on the basis of the median relative abundance of these taxa in the gut microbiome. Patients with high Faecalibacterium abundance had a significantly prolonged PFS versus those with a low abundance (P = 0.03). Conversely, patients with a high abundance of Bacteroidales had a shortened PFS compared to that of those with a low abundance (P = 0.05, Fig. 3D). This is in line with recently published data on CTLA-4 blockade, where patients with a higher abundance of Faecalibacterium had a prolonged PFS compared to those with a higher abundance of Bacteroidales in the gut microbiome (33). In addition, univariate Cox proportional hazards analyses demonstrated that the strongest microbial predictors of response to anti-PD-1 therapy were alpha diversity [intermediate hazard ratio (HR) = 3.60, 95% CI = 1.02 to 12.74; low HR = 3.57, 95% CI = 1.02 to 12.52] and abundance of Faecalibacterium (HR = 2.92, 95% CI = 1.08 to 7.89) and Bacteroidales (HR = 0.39, 95% CI = 0.15 to 1.03) in the fecal microbiome. Our final multivariate model was selected by forward stepwise selection and included Faecalibacterium abundance (HR = 2.95, 95% CI = 1.31 to 7.29, P = 0.03) andprior immunotherapy (HR = 2.87, 95% CI = 1.10 to 7.89, P = 0.03) (table S6). Abundance of Faecalibacterium and Bacteroidales also outperformed relevant clinical variables in receiver operating characteristic curve (ROC) analysis

Next, we sought to gain insight into the mechanism through which the gut microbiome may influence response to anti-PD-1 therapy. We first conducted functional genomic profiling of gut microbiome samples using metagenomic WGS sequencing (n = 25) in R (n = 14) versus NR (n =11). Organism-specific gene hits were assigned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO), and, on the basis of these annotations, metagenomes for each sample were reconstructed into metabolic pathways using the MetaCyc hierarchy of pathway classifications (34, 35). Unsupervised hierarchical clustering of predicted pathway enrichment identified two groups of patient samples, with response rates of 69.2 and 41.7% (Fig. 3E). A similar pattern was also noted for KO abundances with 70.6 and 37.5% response rates (fig. S17). Comparisons of pathway enrichment across these groups showed differences in metabolic functions, with anabolic functions predominating in R-including amino acid biosynthesis (Fig. 3E), which may promote host immunity (36)—and catabolic functions predominating in NR (Fig. 3E, fig. S16, and table S7).

There is clear evidence in preclinical models that differential composition of the gut microbiome may influence therapeutic responses to anti-PD-1 therapy at the level of the tumor microenvironment (12); thus, we next examined the relationship between the gut microbiota and systemic and antitumor immune responses in our cohort of patients on anti-PD-1 therapy. We compared the tumor-associated immune infiltrates using multiparameter immunohistochemistry (IHC) and observed a higher density of CD8⁺ T cells in baseline samples of R versus NR (P =0.04), consistent with prior reports (Fig. 4A and fig. S18) (18, 37). Pairwise comparisons using Spearman rank correlations were then performed between specific bacterial taxa enriched in the gut microbiome of R and NR and immune markers in the tumor microenvironment, demonstrating a statistically significant positive correlation between the CD8⁺ T cell infiltrate in the tumor and abundance of the Faecalibacterium genus, the Ruminococcaceae family, and the Clostridiales order in the gut and a nonsignificant but negative correlation with Bacteroidales (Fig. 4, B and C, and figs. S19 and S20). No associations were seen between CD8+T cell density and diversity or crOTU community type membership (fig. S21). Analysis of systemic immune responses using flow cytometry and cytokine assays revealed that patients with a high abundance of Clostridiales, Ruminococcaceae, or Faecalibacterium in the gut had higher frequencies of effector CD4+ and CD8⁺ T cells in the systemic circulation with a preserved cytokine response to anti-PD-1 therapy, whereas patients with a higher abundance of Bacteroidales in the gut microbiome had higher frequencies of regulatory T cells (T_{ress}) and myeloid-derived suppressor cells (MDSCs) in the systemic circulation, with a blunted cytokine response (Fig. 4D and figs. S22 and S23). To better understand the influence of compositional differences in the gut microbiome on antigen processing and presentation within the tumor microenvironment, we next performed multiplex IHC targeting the myeloid compartment (38). In these studies, patients with a high abundance of Faecalibacterium in the gut microbiome had a higher density of immune cells and markers of antigen processing and presentation compared to that of patients with a high abundance of Bacteroidales (Fig. 4, E and F, and figs. S24 and S25), suggesting a possible mechanism through

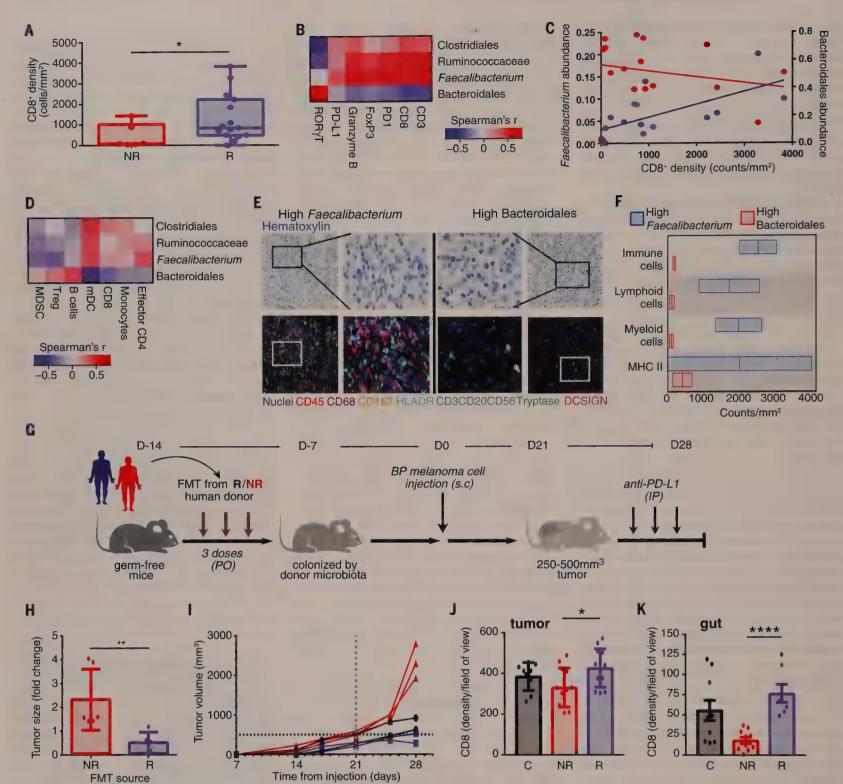


Fig. 4. A favorable gut microbiome is associated with enhanced systemic and antitumor immunity. (A) Quantification by IHC of the CD8⁺ T cell infiltrate at pretreatment in tumors in R (n = 15, blue) and NR (n = 6, red) by one-sided MW test. Error bars represent the distribution of CD8⁺ T cell densities. (B) Pairwise Spearman rank correlation heatmap of significantly different taxa in fecal samples (n = 15) at baseline and CD3, CD8, PD-1, FoxP3, Granzyme B, PD-L1, and RORyT density in matched tumors. (C) Univariate linear regression between CD8⁺ T cell density in counts per mm² in the tumor versus Faecalibacterium [blue, coefficient of determination $(R^2) = 0.42$, P < 0.01] and Bacteroidales (red, $R^2 = 0.06$, P = 0.38) abundance in the gut. (D) Pairwise Spearman rank correlation heatmap between significantly different fecal taxa and frequency of indicated cell types by flow cytometry in peripheral blood at baseline. mDC, myeloid dendritic cell. (E) Representative multiplex IHC images and (F) frequency of various immune cell types in patients having high Faecalibacterium (n = 2) or Bacteroidales (n = 2) in the gut. In (E), rectangles identify magnified region. MHC II, major histocompatibility complex II. (G) Experimental design of studies in germ-free (GF) mice. Time in days (indicated as D) relative to tumor injection (2.5 \times 10⁵ to 8 \times 10⁵ tumor cells). PO, per os (orally); BP, BRAF^{V600E}/PTEN^{-/-}; s.c., subcutaneous;

IP, intraperitoneal. (H) Difference in size by MW test of tumors at day 14, implanted in R-FMT (blue) and NR-FMT mice (red), expressed as fold change (FC) relative to average tumor volume of control GF mice. Data from two independent FMT experiments (R-FMT, n = 5, median FC = 0.18; NR-FMT, n = 6, median FC = 1.52). (I) Representative tumor growth curves for each GF mouse from anti-PD-L1 treated R-FMT (blue, n = 2, median tumor volume = 403.7 mm³), NR-FMT (red, n = 3, median tumor volume = 2301 mm³), and control (black, n = 2, median tumor volume = 771.35 mm³) mice. Statistics are as follows: P = 0.20 (R-FMT versus NR-FMT) and P = 0.33(NR-FMT versus control) by MW test. Dotted black line marks tumor-size cutoff for anti-PD-L1 treatment (500mm³). (J) Quantification of CD8⁺ density in tumor of R-FMT [n = 2, median = 433.5 cells/high-power field](HPF) across 12 regions], NR-FMT (n = 2, median = 325 cells/HPF across 12 regions), and control mice (n = 2, median = 412 cells/HPF across 9 regions). MW test P = 0.30 (R-FMT versus control). (**K**) Quantification of CD8⁺ density in gut R-FMT (n = 2, median = 67 cells/HPF across 7 regions), NR-FMT (n = 2, median = 24 cells/HPF across 5 regions), and control <math>(n = 2, median =47 cells/HPF across 10 regions). MW test P = 0.17 (R-FMT versus control). *P < 0.05; **P < 0.01; ****P < 0.0001.

which the gut microbiome may modulate antitumor immune responses (12), though this must be validated in a larger cohort.

To investigate a causal link between a "favorable" gut microbiome and response to immune checkpoint blockade, we performed fecal microbiota transplantation (FMT) experiments in germfree recipient mice (Fig. 4G). In these studies, mice that were transplanted with stool from responders to anti-PD-1 therapy (R-FMT) had significantly reduced tumor size (P = 0.04; Fig. 4H and fig. S26A) by day 14 compared to those transplanted with stool from NR (NR-FMT). Importantly, mice transplanted with R-FMT stool also exhibited improved responses to anti-PD-L1 (PD-1 ligand 1) therapy (Fig. 4I) in contrast to mice that were transplanted with stool from NR (NR-FMT). Next, we performed 16S sequencing on fecal samples collected from mice treated with FMT, demonstrating that mice transplanted with R-FMT stool also had significantly higher abundance of Faecalibacterium in their gut microbiome (P < 0.01) (fig. S27). We also wanted to better understand the mechanism through which the gut microbiome may influence systemic and antitumor immune responses, and so we performed correlative studies on tumors, peripheral blood, and spleens from these mice. These studies demonstrated that tumors of mice receiving R-FMT stool had a higher density of CD8⁺ T cells than mice receiving NR-FMT, consistent with human data (Fig. 4J and fig. S26B, top series). Analysis of CD45+ myeloid and lymphoid tumor-infiltrating cells by flow cytometry confirmed this result (fig. S26C). Moreover, FMT from R locally increased the number of CD45⁺ immune and CD8⁺ T cells in the gut compared to FMT from NR (Fig. 4K and fig. S26B, bottom series). Mass cytometry analysis using t-distributed stochastic neighbor embedding dimension reduction was performed on tumors from mice and demonstrated up-regulation of PD-L1 in the tumor microenvironment of mice receiving R-FMT versus NR-FMT stool (fig. S26D), suggesting the development of a "hot" tumor microenvironment. Further phenotypic studies of tumor immune infiltrates revealed a significant enrichment of innate effector cells (expressing CD45+CD11b+Ly6G+) in mice receiving R-FMT stool (fig. S26E). A lower frequency of suppressive myeloid cells (expressing CD11b⁺CD11c⁺) was observed in mice receiving R-FMT stool compared to that of mice receiving NR-FMT (fig. S26F). Finally, an increase in the frequency of RORγT+ T helper 17 cells in the tumor was also detected in mice transplanted with NR-FMT stool (fig. S26G), in line with what we observed in tumors from patients who failed to respond to anti-PD-1 therapy. Mice receiving NR-FMT stool also had higher frequencies of regulatory CD4⁺FoxP3⁺ T cells (fig. S26H) and CD4⁺IL-17⁺ T cells (fig. S26I) in the spleen, suggesting impaired host immune responses.

Our results indicate that the gut microbiome may modulate responses to anti-PD-1 immunotherapy in melanoma patients. We propose that patients

with a favorable gut microbiome (for example, high diversity and abundance of Ruminococcaceae and Faecalibacterium) have enhanced systemic and antitumor immune responses mediated by increased antigen presentation and improved effector T cell function in the periphery and the tumor microenvironment. By contrast, patients with an unfavorable gut microbiome (for example, low diversity and high relative abundance of Bacteroidales) have impaired systemic and antitumor immune responses mediated by limited intratumoral lymphoid and myeloid infiltration and weakened antigen presentation capacity. These findings highlight the therapeutic potential of modulating the gut microbiome in patients receiving checkpoint blockade immunotherapy and warrant prompt evaluation in cancer patients through clinical trials.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/97/suppl/DC1 Materials and Methods Figs. S1 to S28 Tables S1 to S9 References (39–79)

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CANCER IMMUNOTHERAPY

The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients

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Anti-PD-1-based immunotherapy has had a major impact on cancer treatment but has only benefited a subset of patients. Among the variables that could contribute to interpatient heterogeneity is differential composition of the patients' microbiome, which has been shown to affect antitumor immunity and immunotherapy efficacy in preclinical mouse models. We analyzed baseline stool samples from metastatic melanoma patients before immunotherapy treatment, through an integration of 16S ribosomal RNA gene sequencing, metagenomic shotgun sequencing, and quantitative polymerase chain reaction for selected bacteria. A significant association was observed between commensal microbial composition and clinical response. Bacterial species more abundant in responders included Bifidobacterium longum, Collinsella aerofaciens, and Enterococcus faecium. Reconstitution of germ-free mice with fecal material from responding patients could lead to improved tumor control, augmented T cell responses, and greater efficacy of anti-PD-L1 therapy. Our results suggest that the commensal microbiome may have a mechanistic impact on antitumor immunity in human cancer patients.

he therapeutic efficacy of immunotherapies targeting the PD-1/PD-L1 interaction is favored in patients who show evidence of a T cell-inflamed tumor microenvironment at baseline (1, 2). Therefore, host and tumor factors that regulate the magnitude of endogenous immune priming and T cell infiltration into the tumor microenvironment are being sought as an opportunity to further expand therapeutic efficacy (3). Preclinical studies have indicated that the composition of the commensal microbiome could exert a major influence; mice with favorable microbiota showed far greater therapeutic activity of anti-PD-L1 treatment than did mice with an unfavorable microbiome, and this benefit could be transferred by cohousing or fecal transplant (4). These observations prompted an analogous analysis of the human microbiome with respect to therapeutic efficacy of anti-PD-1 in cancer patients.

To evaluate whether commensal bacterial composition might be associated with clinical efficacy of PD-1 blockade immunotherapy, stool samples were collected from 42 patients before treatment as part of a multidimensional biomarker analysis in metastatic melanoma. The majority of patients received an anti-PD-1 regimen; four patients received anti-CTLA-4 treatment, but the downstream data conclusions did not change with the removal of these subjects, so they were retained in the analysis. Clinical response rate was determined

in a blinded manner from biomarker results by using Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1. There were 16 responders (from here on, referred to as R) and 26 nonresponders (NR), yielding a response rate of 38%, which is in line with published clinical data of anti-PD-1 therapy in metastatic melanoma patients (5, 6). No major differences in patient characteristics were observed in R versus NR, except a borderline difference in prior (but not current) smoking history (table S1).

To determine whether the composition of the commensal microbiota is associated with clinical response, we integrated three methods for DNA sequence-based bacterial identification (fig. S1A). First, using 16S ribosomal RNA (rRNA) gene amplicon sequencing, we identified operational taxonomic units (OTUs) with taxonomic assignment present at different abundance in R versus NR (table S2). We used a Basic Local Alignment Search Tool (BLAST) search of the 16S sequences against the National Center for Biotechnology Information (NCBI) database to reveal potential species-level identities (table S3). Further level of confidence in species identification was gained by matching the OTUs from the 16S data set to species-level identities revealed through metagenomic shotgun sequencing (table S4). We used species-specific quantitative polymerase chain reaction (PCR) for those candidate species having previously validated primers (table S5). Compared with the 16S analysis, the metagenomic sequencing yielded a smaller number of species differentially represented in R versus NR, which overlapped with the 16S results (table S6). Treating these assays as a screen for maximizing the number of candidate species, we used the 16S sequencing method as a starting point in our analysis.

After removing OTUs present in less than 10% of the samples, the 16S sequencing revealed 62 OTUs of different abundance in R versus NR [P < 0.05, unadjusted, permutation test with]Quantitative Insights Into Microbial Ecology (QIIME)] (table S2). Hierarchical clustering of samples based on relative abundance of these OTUs revealed that most patients were accurately grouped according to clinical response (fig. S2). Clustering of patients within each clinical group is depicted in Fig. 1A. Thirty-nine OTUs were more abundant in R, and 23 were more abundant in NR. One Bifidobacteriaceae OTU was significantly more abundant in R, and a second Bifidobacteriaceae OTU (559527) had borderline significance (P = 0.058, unadjusted) and was included in the analyses (total = 63 OTUs). This observation recapitulates our previous findings that associated Bifidobacteriaceae family members with improved immune-mediated tumor control and efficacy of anti-PD-L1 therapy in mice (4). A principal component analysis (PCA) of the 63 OTUs revealed separation of R from NR (Fig. 1B).

A BLAST search of the 63 OTUs against the NCBI database of bacterial sequences returned for most OTUs multiple species with ≥98% identity (table S3). To gain more accurate specieslevel characterization, the same samples were subjected to metagenomic shotgun sequencing (available for 39 of the 42 samples). Illumina paired-end reads were assigned to microbial clades and analyzed for closest matches to the 63 OTUs identified with 16S sequencing. Potential species matches were identified for 43 of the original 63 OTUs (table S4). Species-specific quantitative PCR assays were performed as an additional approach to assess the identity of species, for which sufficiently validated quantitative PCR primers were available (table S5). Thus, integration of the three methods led to the selection of 10 species differentially enriched in R versus NR. Eight of these were more abundant in R-Enterococcus faecium. Collinsella aerofaciens, Bifidobacterium adolescentis, Klebsiella pneumoniae, Veillonella parvula, Parabacteroides merdae, Lactobacillus sp., and Bifidobacterium longum—whereas two were more abundant in NR: Ruminococcus obeum and Roseburia intestinalis. As an example, the integrative analysis for B. longum (OTU 559627) is depicted in Fig. 2, A to C. Similar correlation analyses for the remaining nine species are depicted in figs. S3 and S4. Quantitative PCR results for these 10 species were integrated into a summation quantitative PCR score for each patient, which was significantly higher in responders (P = 0.004) (Fig. 2D).

This list of species is likely an underestimate of the total number of entities showing differential abundance in R versus NR because of the stringency of this composite analysis. For example, Akkermansia muciniphila OTU (185186), in line with the study of anti-PD-1 efficacy in epithelial cancers by Routy et al. (7), was detected by means of 16S sequencing in four patients, and all were responders, but statistical analysis of the entire cohort is limited by the number of samples above the detection threshold. As an alternative way to

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represent the aggregate data toward development of a candidate predictive biomarker, the total numbers of potentially "beneficial" and "nonbeneficial" OTUs were scored for each patient (fig. S5), and a ratio was calculated. When plotted against the absolute change in tumor size as assessed with RECIST, a clean correlation was observed so that patients with a ratio over 1.5 all showed clinical response (Fig. 2E). These results suggest that the commensal microbiota composition might be useful as a biomarker to predict response to checkpoint blockade therapy, which motivated comparison with other candidate predictive biomarkers. Archived pretreatment tumor specimens that passed quality control were available for 15 patients (5 R, 10 NR). Microbial composition remained significantly different in R versus NR for this subset (P < 0.01) (fig. S6, A and B). Exome sequencing followed by enumeration of nonsynonymous somatic mutations showed a trend of higher frequency in R, as did levels of PD-L1 and PD-1 mRNA (fig. S6, C to E) and enumeration of baseline CD8⁺ T cells by means of immunohistochemistry (fig. S6F). Although these trends not reaching statistical significance level of 0.05 were likely limited by sample size, the microbiota parameters still markedly separated responders and nonresponders.

The strong correlation between commensal bacteria and clinical response to immunotherapy suggested a potential causal effect, in light of data demonstrating an immune-potentiating impact of the microbiome in mouse tumor models (4, 8–10). To investigate the capability of human commensal microbes to potentiate antitumor T cell responses, we used germ-free (GF) mice as recipients. We had previously reported that spontaneous immunemediated tumor control in Taconic mice could be improved by means of fecal microbiota transfer from mice obtained from a different vendor, the Jackson Laboratories (4). In setting up the current model, we found that B16.SIY melanoma tumor growth in GF mice was similar to that in specific pathogen-free (SPF) mice (both from Taconic), and colonization of GF mice with feces from Taconic SPF mice did not affect this baseline growth rate (fig. S8). These results suggest a reduced spontaneous immune-mediated tumor control inherent to GF mice, which makes them suitable recipients for human-derived microbiota, with an opportunity to detect improved antitumor immunity depending on microbial composition. Fecal material was transferred from three R and three NR into cohorts of GF mice (Fig. 1A and figs. S2, S5, and S7), followed by implantation of B16.SIY melanoma cells 2 weeks later. The human microbiota-colonized mouse groups segregated into two phenotypes with respect to tumor growth rate: (i) a faster growing group and (ii) a slower growing group (Fig. 3A). Two of three mouse cohorts reconstituted with R fecal material had slower baseline tumor growth, and two of the three cohorts reconstituted from NR showed faster baseline tumor growth. Thus, the ability of the human microbiota to support improved tumor control in mice usually, but not always, paralleled the clinical response to

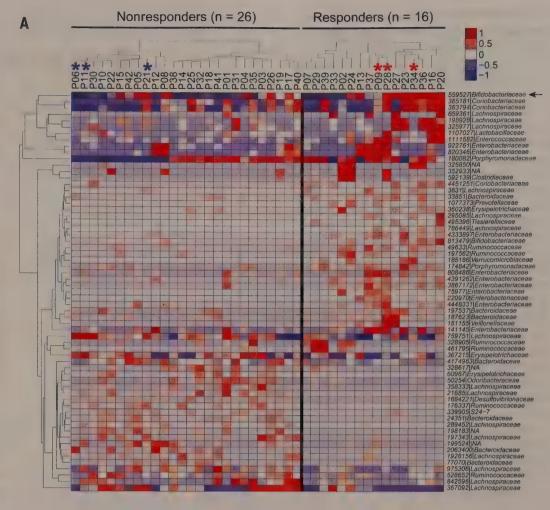
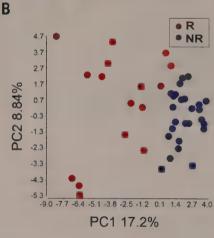


Fig. 1. Distinct commensal microbial communities in anti-PD-1 responding patients and nonresponding patients as assessed with 16S rRNA gene amplicon sequencing. (A) Relative abundance of differentially abundant taxa in responders versus nonresponders; 62 OTUs were identified as different with P < 0.05 (unadjusted, permutation test). An additional OTU 559527 (arrow) identified as Bifidobacteriaceae approached significance (P = 0.058). Hierarchical clustering of the samples was performed within each clinical group. Individual samples are organized in columns, labeled with patient identification number. Asterisks indicate samples used in further in vivo experiments. The ID of de novo assembled OTUs (new clean-up reference OTUs picked with QIIME) were



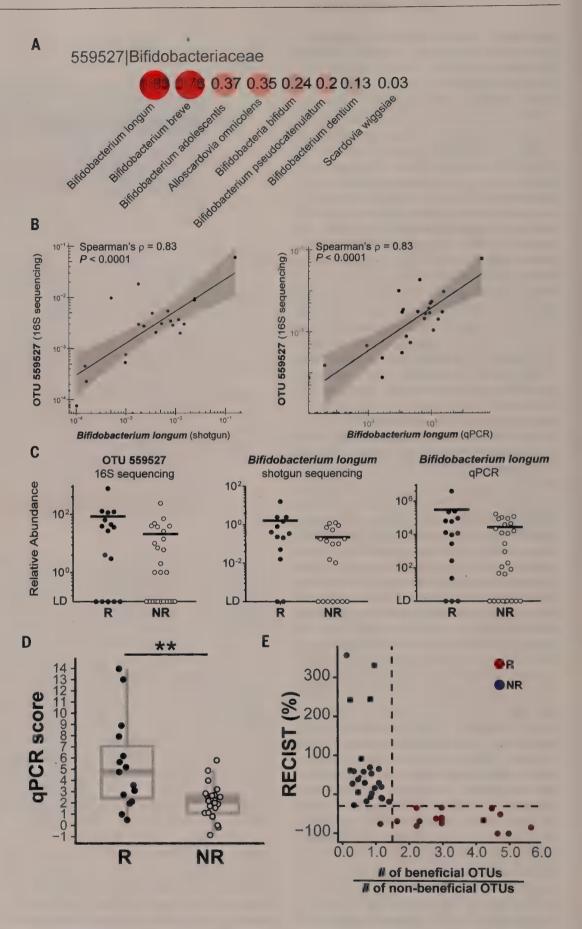
abbreviated to show only the individual identifier digits, and the full OTU IDs are provided in table S4. (B) PCA of relative abundance of the 63 OTUs shown in Fig. 1A.

anti-PD-1 seen in the donor patient. Achieving slower tumor growth with fecal transplant alone is similar to previous mouse studies, in which transfer of feces from Jackson into Taconic mice was sufficient for a partial therapeutic effect owing to a more favorable microbiome (4).

Composition of bacterial taxa that successfully reconstituted mice and fidelity to the original human donor were assessed with 16S rRNA gene amplicon sequencing. Groups C and D, which did not show the same pattern of tumor control as the therapeutic outcome in the original human donors, showed a large degree of difference of microbiota composition from the original human donors (fig. S9). In agreement, a binary Bray-Curtis dissimilarity index for each donor/recipient pair was highest, at 0.7, for cohorts C and D versus 0.5 to 0.6 for the rest of the groups. We conclude that whereas reconstitution of GF mice with human fecal material often recapitulates the microbial composition and the phenotype of the human donor, in some cases there is a high degree of drift so that some bacteria expand and others contract to a degree that is sufficient to change phenotype. Nonetheless, for the reconstituted GF mice that do recapitulate the clinical outcome of the original donor, this model system may be useful for the ultimate isolation of specific bacteria that regulate antitumor immunity in vivo.

We focused on mouse groups A and B for further mechanistic studies. There was a high level of consistency between repeated experiments,

Fig. 2. Identification of commensal bacterial species associated with patient clinical response to anti-PD-1 therapy. (A) Spearman's correlation coefficients between the relative abundances of Bifidobacteriaceae OTU 559527 from the 16S data set and species-level identities suggested by shotgun sequencing. The species profiled with shotgun sequencing were compared with the taxonomy of OTUs generated from 16S sequencing at the family level. (B) Spearman's correlation between abundance of OTU 559527 from the 16S data set and B. longum identified by means of metagenomics shotgun sequencing analysis (left) and quantitative PCR (right). Shaded band indicates 95% confidence interval (CI) of the values fitted by linear regression. (C) Relative abundance in responders (R) versus nonresponders (NR) of OTU 559527 (16S sequencing; left), B. longum (shotgun sequencing; middle), and B. longum (quantitative PCR; right), LD, limit of detection. (D) Quantitative PCR score representing aggregate data for the relative abundances of 10 species correlated to OTUs with differential abundance in R versus NR. Wilcoxon-Mann-Whitney test (nonparametric) was used to compare quantitative PCR score between R and NR groups. (E) Ratio of beneficial to nonbeneficial OTU numbers for each patient versus the patient's RECIST aggregate tumor measurement change. Dashed lines label RECIST% = -30 and ratio = 1.5. Only the 43 16S OTUs confirmed with shotgun metagenomic sequencing were included. P < 0.05 was considered statistically significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.



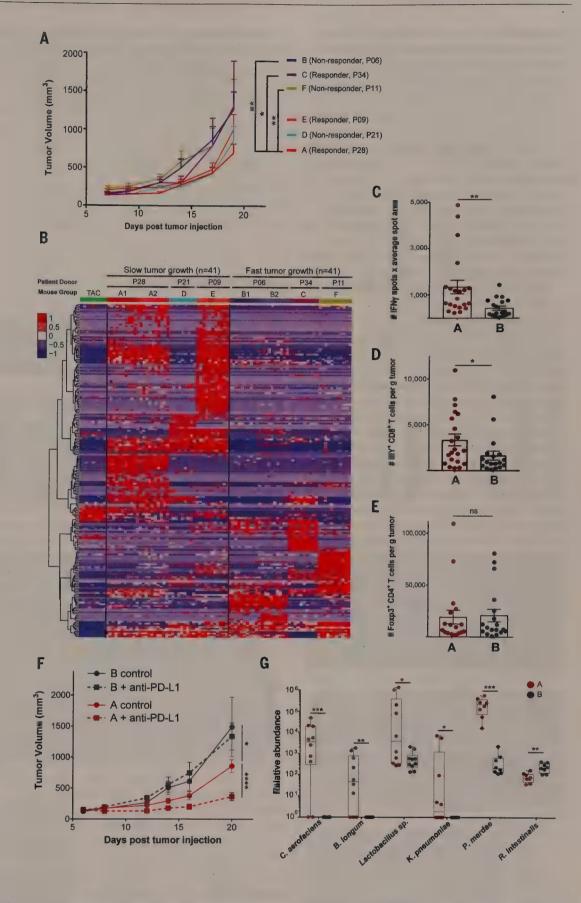
both with respect to tumor growth rate and microbial colonization (Fig. 3B, group A1 versus A2 and B1 versus B2 comparisons). To determine whether the difference in tumor control could be attributed to host immunity, interferon-γ (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) of ex vivo SIY-stimulated splenocytes was performed and indicated an increased frequency of activated T cells from R microbiota-reconstituted mice 3 weeks after inoculation with B16.SIY melanoma cells (Fig. 3C). Analysis of the tumor micro-

environment also showed a significantly greater number of SIY-specific CD8⁺ T cells, but not of FoxP3⁺CD4⁺ regulatory T cells, in these mice (Fig. 3, D and E), which is consistent with increased priming of tumor antigen-specific CD8⁺ T cells. Anti-PD-L1 was markedly efficacious in mice colonized with R microbiota yet completely ineffective in NR-derived mice (Fig. 3F), demonstrating a profound impact of the commensal microbiota on immunotherapy efficacy in vivo. Interrogation of fecal DNA from these mice by means of

quantitative PCR recapitulated the results from our analysis of patients. Of the PCR reactions validated in patients, six were observed in reconstituted mice, with the same pattern of enrichment as was seen in patients (Fig. 2G).

Together, our data suggest that the composition of the commensal microbiota in patients is associated with therapeutic efficacy of anti-PD-1 monoclonal antibody (mAb). Although *B. longum* was one commensal identified in the current study that had also been found in mouse

Fig. 3. Human commensal communities modulate antitumor immunity in mouse melanoma model. GF mice were gavaged with fecal material from three responder (P28, P34, and P09) and three nonresponder (P06, P21, and P11) patient donors. (A) B16. SIY melanoma was injected subcutaneously 2 weeks after gavage; tumor growth data are from one (groups C, D, E, and F) or two experiments (groups A and B) with 7 to 11 mice per group per experiment. Error bars represent mean + SEM. (B) Relative abundance of 207 OTUs from patient donors that colonized in mice and were differentially abundant between slow- and fast-tumorgrowth groups. Columns depict individual mice arranged in groups A through F. Groups A1, B1, A2, and B2 are from two independent duplicate experiments. Rows indicate individual OTUs with exact reference ID match between human and mouse 16S rRNA data sets. (C) In groups A and B, ex vivo activation of splenocytes by SIY peptide was measured with IFN-y ELISPOT 3 weeks after tumor injection. (D and E) Tumor-infiltrating SIY-specific CD8+ T cells (D) and FoxP3+ regulatory T cells (E) were enumerated with flow cytometry. (F) Efficacy of anti-PD-L1 therapy was determined in groups A and B. Data are from one experiment with 7 or 8 mice per group. (G) Relative abundance in mouse groups A and B of key species validated for quantitative PCR scoring. Six out of the 10 species are shown that gave positive PCR signals. The remaining four species were absent from these particular recipient groups. Tumor growth curves were analyzed with two-way analysis of variance by Tukey's multiple comparisons post-test; flow cytometry and quantitative PCR data were analyzed by Wilcoxon-Mann-Whitney test (nonparametric). P < 0.05 was considered statistically significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.



models to be associated with improved immunemediated tumor control (4), it seems likely that multiple specific bacteria may contribute to improved antitumor immunity in patients. In addition to the panel of bacteria overrepresented in responders, several OTUs were overrepresented in nonresponders, and prior work in mice has indicated that some commensals have the potential to be immune-inhibitory—for example, through the induction of FoxP3⁺ regulatory T cells (11, 12). In addition, our current cohort suggested that a ratio of "beneficial" OTUs to "nonbeneficial" OTUs was the strongest predictor of clinical response. This may indicate that a higher frequency of beneficial bacteria, together with a lower frequency of bacteria with negative impact, may combine for the most favorable clinical outcome.

Several of the bacterial species that were identified in the current study to be differentially abundant in responding versus nonresponding patients have been examined previously for mechanistic impact on host immune responses in GF mice in vivo (13). Monocolonization with several species found to be at increased frequency in our responders—including E. faecium, C. aerofaciens, B. adolescentis, and P. merdae—were reported to result in a decreased frequency of peripherally derived colonic regulatory T cells as compared with that of other bacterial species. An increased frequency of the Batf3-lineage dendritic cells (DCs) and greater T helper cell 1 (T_H1) responses were also found with bacteria currently identified to be more abundant in responders (13). Decreased regulatory T cells, increased Batf3 DCs, and augmented THIresponses would all be expected to improve immune-mediated tumor control. Although care should be taken extrapolating these results in the setting of a complex microbiota, the data suggest that patient responder-associated bacteria may have a distinct effect on innate and adaptive immunity both locally and systemically.

Two additional studies have identified an association of the commensal microbiome with anti-PD-1 mAb efficacy in different solid cancers (7, 14). Our approaches differ in the method of segregation of patients between R and NR groups and in some methods of analysis. Therefore, direct comparison of the differentially enriched commensal microbiota in R and NR patients across publications should be addressed with caution. Nonetheless, there is agreement with respect to a mechanistic impact of the microbiome on efficacy of anti-PD-1 immunotherapy because fecal transfer from R versus NR patients to GF mice was also able to recapitulate the patient phenotype.

In addition to the microbiome, it is apparent that additional tumor and host factors can affect the efficacy of antitumor immunity and cancer immunotherapy. Tumor-intrinsic activation of the Wnt/β-catenin pathway (15) and deletion or mutation of Pten (16) have been shown to lead to deficient T cell infiltration into the tumor microenvironment and resistance to checkpoint blockade immunotherapy. Germline polymorphisms in immune regulatory genes also have the potential to influence the magnitude of spontaneous antitumor T cell responses (17). Our results described here open the avenue for integrating commensal microbial composition, along with tumor genomics and germline genetics, into a multiparameter model with which to maximize the ability of predicting which patients are likely to respond to immunotherapies such as anti-PD-1.

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US20160354416 A1 submitted by the University of Chicago that covers the use the microbiota to improve cancer immunotherapy. J.J.L. is a consultant to and receives research funding from Bristol-Myers Squibb and Merck.

SUPPLEMENTARY MATERIALS

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MOLECULAR BIOLOGY

A mitosis-specific and R loop-driven ATR pathway promotes faithful chromosome segregation

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The ataxia telangiectasia mutated and Rad3-related (ATR) kinase is crucial for DNA damage and replication stress responses. Here, we describe an unexpected role of ATR in mitosis. Acute inhibition or degradation of ATR in mitosis induces whole-chromosome missegregation. The effect of ATR ablation is not due to altered cyclin-dependent kinase 1 (CDK1) activity, DNA damage responses, or unscheduled DNA synthesis but to loss of an ATR function at centromeres. In mitosis, ATR localizes to centromeres through Aurora A-regulated association with centromere protein F (CENP-F), allowing ATR to engage replication protein A (RPA)-coated centromeric R loops. As ATR is activated at centromeres, it stimulates Aurora B through Chk1, preventing formation of lagging chromosomes. Thus, a mitosis-specific and R loop-driven ATR pathway acts at centromeres to promote faithful chromosome segregation, revealing functions of R loops and ATR in suppressing chromosome instability.

he ataxia telangiectasia mutated and Rad3related (ATR) kinase is a master checkpoint regulator safeguarding the genome (1). Loss of ATR during DNA replication increases genomic instability in S phase and subsequent mitosis (2-4). While analyzing the localization of ATR in diploid retinal pigment epithelium 1 (RPE1) cells across the cell cycle, we unexpectedly found that ATR localized to all centromeres detected by ACA (anticentromere antibody) on mitotic chromosomes (Fig. 1, A and B, and fig. S1A). Autophosphorylated ATR (p-ATR T1989) also colocalized with ACA (Fig. 1A and fig. S1B), suggesting that ATR is active at centromeres. The staining of ATR and p-ATR was eliminated by ATR small interfering RNA (siRNA) (fig. S1, C and D), confirming the specificity of the antibodies. Colocalization of ATR and centromere protein B (CENP-B) was detected by the proximity ligation assay in unperturbed mitotic cells but not in inter-

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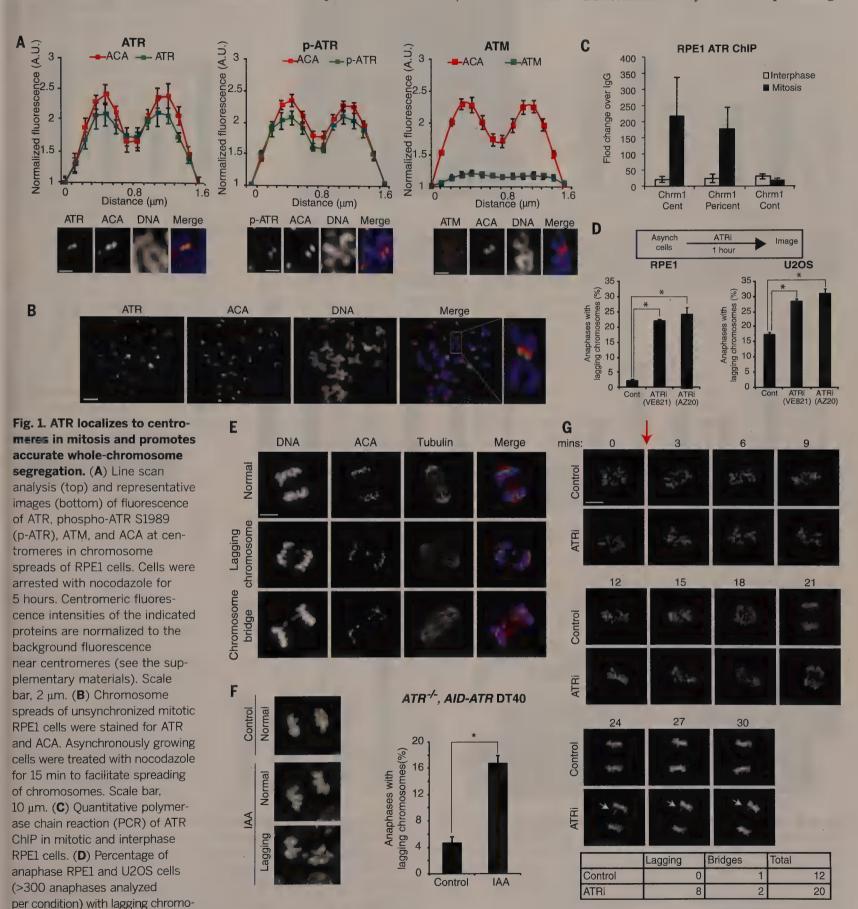
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phase cells (fig. S2, A and B). Chromatin immunoprecipitation (ChIP) of ATR confirmed that ATR was enriched at the centromere of chromosome 1 compared with a noncentromere locus in mitotic RPE1 and U2OS cells (Fig. 1C and fig. S2C). In contrast to ATR, another master checkpoint kinase ATM was not detected at centromeres (Fig. 1A).

The presence of ATR at centromeres during mitosis prompted us to investigate whether ATR functions in chromosome segregation. Short (1 hour) treatments of asynchronously growing RPE1 and U2OS cells with two structurally distinct ATR inhibitors (ATRi), VE-821 and AZ20, increased the rate of lagging chromosomes in anaphase (Fig. 1, D and E), indicating a defect in wholechromosome segregation (5, 6). Rapid degradation of auxin-inducible degron (AID)-tagged ATR in mitotic ATR^{-/-}, AID-ATR DT40 cells also increased lagging chromosomes (Fig. 1F and fig. S3A) (7). In contrast to lagging chromosomes, anaphase bridges typically arise from S-phase problems persisting into mitosis but not wholechromosome missegregation (8). Short ATRi treatments did not induce anaphase bridges (fig. S3B), suggesting that the S-phase cells affected by ATRi had not entered mitosis. As ATRi-treated mitotic cells progressed into the next cell cycle, an increased rate of an euploidy was observed (fig. S3C). These results suggest that ATR has a previously uncharacterized role in chromosome segregation distinct from its functions in S phase.

Replication interference in S phase may affect mitosis indirectly by inducing persistent DNA synthesis in mitotic cells (fig. S4, A and B) (9). To investigate whether ATR has a replication-independent role in mitosis, we arrested RPE1

and U2OS cells in G2 with cyclin-dependent kinase I (CDKI) inhibitor and then released them into mitosis in the absence or presence of ATRi (fig. S4, A and B). Postreplication ATRi treatment did not induce DNA synthesis or anaphase bridges



somes after mock or ATRi ($10\,\mu\text{M}$ VE-821, $1\,\text{hour}$) treatments. (**E**) Representative images of anaphase RPE1 cells in (D). Scale bar, $5\,\mu\text{m}$. (**F**) $ATR^{-/-}$, AID-ATR DT40 cells were untreated or treated with 0.5 mM indole-3-acetic acid (IAA) for 30 min to induce AID-ATR degradation. Representative maximum projection images of anaphase cells (left) and quantification of cells with

lagging chromosomes (right) are shown (>300 anaphases analyzed per condition). Scale bar, 5 μ m. (**G**) Time-lapse imaging of H2B-GFP–expressing RPE1 cells untreated or treated with ATRi. Red arrow signifies time of treatment. A lagging chromosome is demarcated by white arrows. Scale bar, 5 μ m. Error bars in all panels represent mean \pm SEM. *P < 0.01; two-tailed t test.

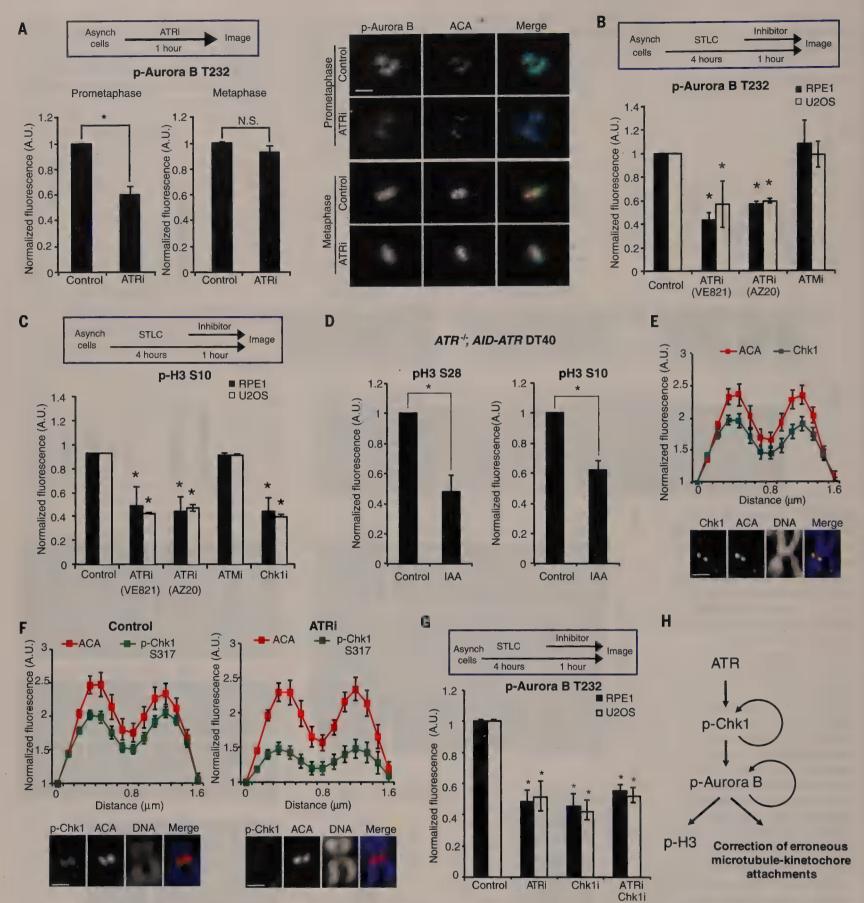


Fig. 2. ATR promotes Aurora B activation at centromeres.

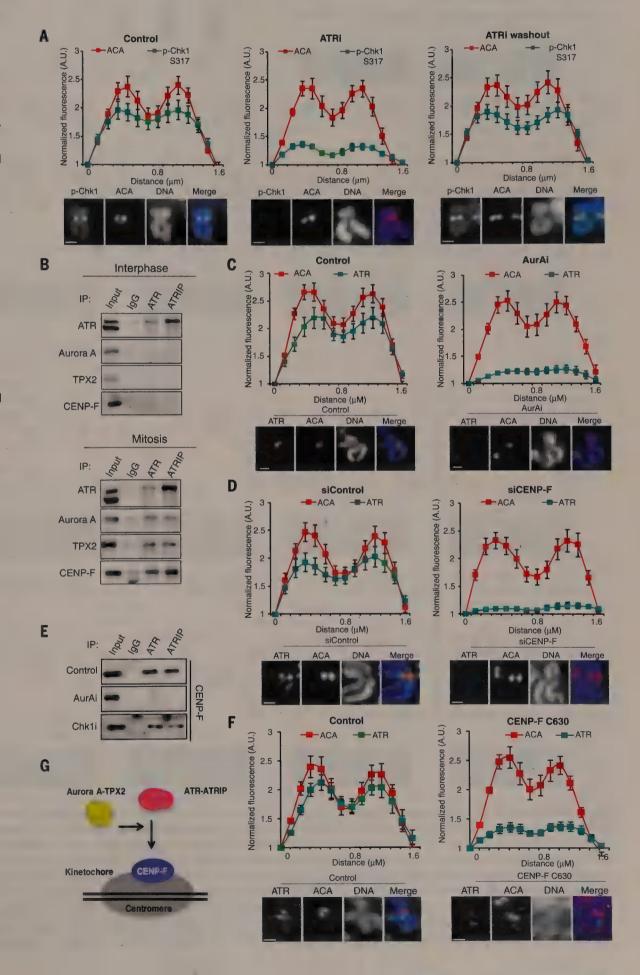
(**A**) Fluorescence intensity of phospho-Aurora B T232 (p-Aurora B) at centromeres in prometaphase or metaphase RPE1 cells untreated or treated with ATRi for 1 hour (left). Representative images of untreated and ATRi-treated cells (right). Scale bar, 5 μ m. (**B** and **C**) Fluorescence intensities of centromeric p-Aurora B (B) and overall p-H3 S10 (C) in S-Trityl L-cysteine (STLC)—arrested prometaphase cells after mock treatments or treatments with the indicated inhibitors for 1 hour. (**D**) Fluorescence intensities of p-H3 S28 and p-H3 S10 in $ATR^{-/-}$, AID-ATR DT40 cells untreated or treated with IAA for 30 min. (**E**) Line scan analysis of Chk1 and ACA at centromeres in chromosome spreads of RPE1 cells.

Cells were arrested with nocodazole for 5 hours. Scale bar, 2 μ m. (**F**) Line scan analysis of p-Chk1 and ACA at centromeres in chromosome spreads of RPE1 cells. Cells were arrested with nocodazole for 4 hours and then mock or ATRi treated for 1 hour. Scale bar, 2 μ m. (**G**) Fluorescence intensity of centromeric p-Aurora B in STLC-arrested prometaphase cells after mock treatments or treatments with the indicated inhibitors. Error bars in all panels represent mean \pm SEM. * $P \le 0.01$; two-tailed t test. (**H**) A schema of the ATR-Chk1-Aurora B pathway in mitosis. This pathway is required for full Aurora B activation, which is necessary for H3 phosphorylation and proper kinetochore-microtubule attachment.

but increased lagging chromosomes (fig. S4, B to D). To unequivocally separate the functions of ATR in S phase and mitosis, we treated live prometaphase cells with ATRi and followed chromosome segregation. ATRi treatment of cells already in mitosis increased the rate of lagging chromosomes in anaphase (Fig. 1G), demonstrating that ATR acts in mitosis to ensure faithful chromosome segregation.

In mitotic cells, ATRi did not affect baseline levels of DNA synthesis and yH2AX, and the canonical ATR pathway was not activated by replication inhibitor hydroxyurea (fig. S5, A to C). ATRi did not significantly alter mitotic progression or affect the inhibitory phosphorylation of CDK1 at Y15 (fig. S6, A to C). Furthermore, ATRi did not increase the rate of multipolar spindles in prometaphase or lead to premature mitotic exit (fig. S6, D and E). However, ATRi reduced the autophosphorylation of Aurora B at T232 (p-Aurora B) in prometaphase (Fig. 2, A and B,

Fig. 3. ATR localizes to centromeres in an Aurora A and CENP-Fdependent manner. (A) Line scan analysis (top) and representative images (bottom) of centromeric p-Chk1 and ACA in chromosome spreads of RPE1 cells. Mitotic cells were isolated by shake-off after 4 hours of nocodazole treatment. Cells were then mock treated (control) or treated with 2 µM VE-821 (ATRi) for 1 hour in the presence of nocodazole. The ATRi washout sample was released from VE-821 for 1 hour in the presence of nocodazole. (B) Immunoprecipitates of endogenous ATR and ATRIP from interphase (top) or mitotic (bottom) RPE1 cell extracts. Input = 5%. (C) Line scan analysis (top) and representative images (bottom) of centromeric ATR in chromosome spreads of RPE1 cells. Cells were arrested with nocodazole for 4 hours and then mock or AurAi treated for 1 hour. (D) Line scan analysis of centromeric ATR and ACA in chromosome spreads of RPE1 cells. Cells were treated with control or CENP-F siRNA and arrested with nocodazole for 5 hours. (E) CENP-F in the immunoprecipitates of endogenous ATR and ATRIP from mitotic RPE1 cell extracts. Cells were treated with AurAi and Chkli as indicated. Input = 5%. (F) Line scan analysis (top) and representative images (bottom) of centromeric ATR and ACA in U2OS cells uninfected or infected with CENP-F C630 expressing retrovirus. Scale bar in all panels, 2 μm. Error bars in all panels represent mean \pm SEM. *P \leq 0.01; two-tailed t test. (G) A model in which ATR is recruited to the vicinity of centromeres through an interaction with CENP-F. This interaction is dependent on Aurora A activity and occurs specifically in mitosis.



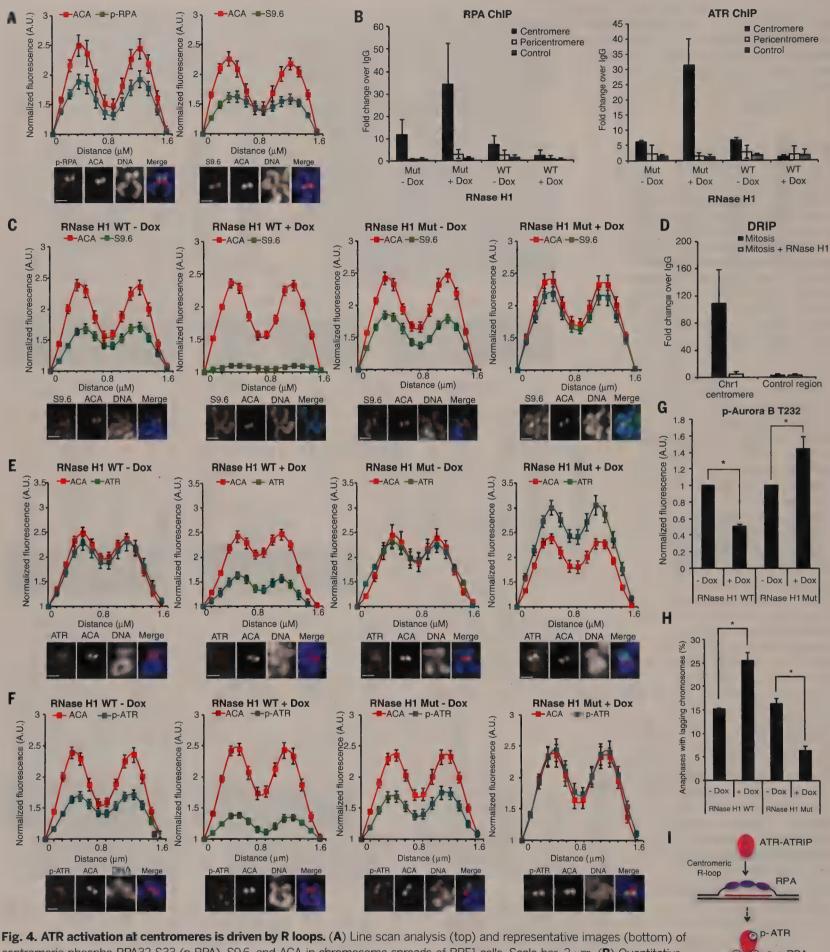


Fig. 4. ATR activation at centromeres is driven by R loops. (A) Line scan analysis (top) and representative images (bottom) of centromeric phospho-RPA32 S33 (p-RPA), S9.6, and ACA in chromosome spreads of RPE1 cells. Scale bar, 2 μm. (B) Quantitative PCR of RPA (right) or ATR (left) ChIP in HeLa-derived RNaseH1 WT/MUT inducible cell lines. Cells were synchronized in G2 with CDK1i, uninduced or induced with Dox for 4 hours, and released into mitosis in the presence of nocodazole for 1 hour (see the supplementary materials). (C, E, and F) Line scan analysis (top) and representative images (bottom) of centromeric S9.6 (C), ATR (E), p-ATR (F), and ACA in RNaseH1 WT/MUT inducible cell lines. Cells were treated as in (B). Scale bar, 2 μm. (D) Quantitative PCR of DRIP in mitotic RPE1 cells. (G) Relative fluorescence intensity of p-Aurora B in RNaseH1 WT/MUT inducible cell lines. Cells were treated as in (B). (H) Percentage of anaphase cells with lagging chromosomes. Cells were treated as in (B). Scale bar for all panels, 2 μm. Error bars in all panels represent mean ± SEM. *P ≤ 0.01; two-tailed t test. (I) A model in which ATR-ATRIP is recruited to and activated by RPA-coated centromeric R loops in mitosis. The red line depicts centromeric RNA hybridized with DNA.

and fig. S7A) (10). ATRi did not affect Aurora B activity in vitro (fig. S7B), ruling out off-target effects on Aurora B. In contrast to ATRi, ATM inhibitor (ATMi) did not affect p-Aurora B (Fig. 2B). Although ATRi reduced p-Aurora B, it did not affect the centromeric localization of Aurora B and other components of the chromosome passenger complex (fig. S7C). Moreover, the activation of Aurora A and polo-like kinase 1 (PLK1) was not affected by ATRi (fig. S7D). The Aurora B-dependent phosphorylation of histone H3 at S10 and S28 was reduced by ATRi (Fig. 2C and fig. S8, A and B). When AID-ATR was rapidly degraded in mitotic $ATR^{-/-}$, AID-ATR DT40 cells, p-H3 S10 and p-H3 S28 was also reduced (Fig. 2D and fig. S8C). Nonetheless, ATRi did not significantly affect the localization of BubR1 to kinetochores (fig. S8D), suggesting that Aurora B retains some of its functions (11). Partial inhibition of Aurora B by AurBi increased the rate of lagging chromosomes (fig. S9, A and B) (12), recapitulating the effects of ATRi. Combination of ATRi with low concentrations of AurBi further decreased p-Aurora B and increased lagging chromosomes (fig. S9, A and B). Thus, ATR is required for the full activation of Aurora B at centromeres, which is critical for correction of erroneous microtubule attachments at kinetochores (Fig. 2H) (12, 13).

To determine how ATR regulates Aurora B in mitosis, we analyzed the ATR effector kinase Chkl, which is implicated in Aurora B activation (14). Like ATR, Chk1 and its ATR- and autophosphorylated forms (p-Chk1 S317, S345, and S296) were detected at all centromeres in mitotic cells (Fig. 2, E and F, and fig. S10, A and B). ATRi reduced p-Chk1 at centromeres (Fig. 2F), suggesting that Chk1 is phosphorylated by ATR. Similar to ATRi, Chk1 inhibitor (Chk1i) did not affect Aurora B activity in vitro but reduced p-Aurora B, p-H3 S10, and p-H3 S28 in mitotic cells (Fig. 2, C and G, and figs. S8B and S10C). Furthermore, the combination of ATRi and Chkli did not further reduce p-Aurora B compared with ATRi or Chkli alone (Fig. 2G), suggesting that ATR promotes Aurora B activation through Chk1 (Fig. 2H).

The unexpected role of ATR at centromeres raises the question as to how ATR is activated. To distinguish whether ATR is activated during or before mitosis, we investigated whether ATR can be reactivated in mitotic cells after transient inhibition. At 2 µM, the ATRi VE-821 inhibited ATR in a reversible manner in interphase cells (fig. S11A). On mitotic chromosomes, the p-Chk1 at centromeres was reduced by 2 µM ATRi but quickly recovered after ATRi washout (Fig. 3A and fig. S11B), showing that ATR is reactivated. Similarly, in mitotic cells p-Aurora B and p-H3 S10 also recovered after ATRi washout (fig. S11, C and D), confirming de novo activation of ATR in mitosis.

To understand how ATR localizes to centromeres in mitosis, we investigated whether ATR interacts with mitotic regulators and centromere proteins. Immunoprecipitations of ATR and its regulatory partner ATR-interacting protein (ATRIP) from mitotic cell extracts but not interphase cell extracts, captured Aurora A, TPX2, and CENP-F (Fig. 3B and fig. S12A). ATR-ATRIP did not capture several other mitotic regulators (fig. S12B), suggesting that ATR transiently but specifically interacts with the Aurora A-TPX2 complex and CENP-F in mitosis. The levels of ATR at centromeres were reduced by AurAi and CENP-F siRNA (Fig. 3, C and D, and fig. S12C). The p-ATR at centromeres was diminished by two independent AurAi, but not by inhibitors of ATM, Chk1, Aurora B, MPS1, and the proteasome inhibitor MG132 (fig. S12D). Importantly, AurAi disrupted the interaction between ATR-ATRIP and CENP-F without altering the levels and localization of CENP-F (Fig. 3E and fig. S12E), suggesting that Aurora A promotes the localization of ATR to centromeres by enabling ATR-ATRIP to bind CENP-F (Fig. 3G). Consistent with the role for CENP-F in ATR localization, a CENP-F fragment that binds to kinetochores exerted dominant negative effects on ATR and p-H3 S10 at centromeres (Fig. 3F and fig. S13) (15). Thus, Aurora A and CENP-F likely bring ATR-ATRIP to the vicinity of centromeres in mitosis, facilitating its activation at centromeres (Fig. 3G).

We next investigated how ATR is activated at centromeres in mitosis. The single-stranded DNA (ssDNA) binding protein RPA (replication protein A) directly interacts with ATRIP and plays a key role in ATR activation at sites of DNA damage and stalled replication forks (16). Although DNA damage and DNA synthesis were undetectable at centromeres in mitosis (9), p-RPA staining was detected at all centromeres in synchronized and unsynchronized mitotic cells (Fig. 4A and fig. S14, A and B). The RPA at centromeres was phosphorylated at S33, a substrate site of ATR. Like ATR, RPA was detected at the centromere of chromosome 1, but not a noncentromere locus, by ChIP (Fig. 4B and fig. S14C). ssDNA is not only generated by DNA repair and DNA replication but also by R loops, which contain DNA-RNA hybrids and displaced ssDNA (17, 18). In yeast, R loops are found at centromeres and linked to H3 S10 phosphorylation and chromatin compaction (19). We recently showed that RPA is a sensor of R loops (20), raising the possibility that RPA is recruited to centromeres by the ssDNA in R loops.

To determine whether R loops are present at centromeres in human cells, we analyzed mitotic chromosomes in RPE1 cells with the monoclonal antibody S9.6, which specifically recognizes DNA-RNA hybrids (18). Indeed, S9.6 staining was detected at all centromeres in synchronized and unsynchronized mitotic cells (Fig. 4A and fig. S15, A and B). S9.6 staining was enriched at centromeres compared with telomeres and chromosome arms (fig. S15C). To confirm that centromeric S9.6 signals arise from DNA-RNA hybrids, we conditionally expressed ribonuclease H1 (RNaseH1), an enzyme that cleaves the RNA in DNA-RNA hybrids, in HeLa cells. The centromeric S9.6 staining was reduced by induction of wild-type RNaseH1 (RNaseH1WT) (Fig. 4C). In contrast, the catalytically inactive RNaseH1 D210N mutant (RNaseH1MUT) (21), which is dominant negative over endogenous RNaseH1, increased S9.6 staining at centromeres (Fig. 4C). RNaseA, which cleaves single- and doublestranded RNA, did not affect S9.6 staining at

centromeres (fig. S15D). Consistent with S9.6 staining, R loops were detected at the centromere of chromosome 1, but not a noncentromere locus, by DNA-RNA immunoprecipitation (DRIP) (Fig. 4D). The DRIP signal at the centromere was abolished by RNaseH1. Thus, like ATR, R loops are specifically enriched at centromeres in mitosis.

Next, we investigated whether R loops regulate RPA and ATR at centromeres in mitosis. The ChIP signals of RPA and ATR at the centromere of chromosome 1 were reduced by RNaseH1WT but enhanced by RNaseH1MUT (Fig. 4B). Furthermore, the ATR staining at centromeres was decreased by RNaseH1WT but increased by RNaseH1MUT (Fig. 4E). The centromeric p-ATR and p-RPA staining was also diminished by RNaseH1WT but enhanced by the RNaseH1MUT (Fig. 4F and fig. S16A), suggesting that R loops are important for ATR activation at centromeres (Fig. 4I). The levels of CENP-F at kinetochores were only slightly affected by RNaseH1 (fig. S16B), suggesting that R loops largely act downstream of CENP-F to promote ATR activation. RNA polymerase II (RNAPII)mediated transcription occurs at centromeres in mitosis and is required for accurate chromosome segregation (22, 23). Inhibition of RNAPII but not RNA polymerase III in mitotic cells reduced the ATR and p-RPA at centromeres (fig. S16C), suggesting that centromeric R loops are generated through RNAPII-mediated transcription during mitosis.

The role of R loops in ATR activation at centromeres predicts that R loops are important for Aurora B activation and suppression of lagging chromosomes. Indeed, p-Aurora B was reduced by RNaseH1WT but increased by RNaseH1MUT (Fig. 4G). Furthermore, the rate of lagging chromosomes was increased by RNaseH1WT but reduced by RNaseH1^{MUT} (Fig. 4H). Notably, RNaseH1^{MUT} increased p-H3 S10 and reduced lagging chromosomes, and both of these effects were reversed by ATRi (fig. S17, A and B), suggesting that stabilization of R loops facilitates Aurora B activation and chromosome segregation through ATR. Together these results strongly suggest that the R loop-ATR circuitry is important for accurate chromosome segregation.

Our results revealed a mitosis-specific ATR pathway that is distinct from the canonical ATR pathway operating in S phase and the DNA damage response. The activation of the mitotic ATR pathway is independent of DNA damage and DNA replication but dependent on the key mitotic regulator Aurora A and R loops at centromeres (fig. S18). In an Aurora A-dependent manner, ATR interacts with CENP-F in mitosis and is brought to the vicinity of centromeres. The R loops at centromeres may present RPA-coated ssDNA, a key platform for ATR activation. Unlike the canonical ATR pathway, the mitotic ATR pathway avoids the suppression by centromeric DNA loops behind replication forks (24). The requirement of both the Aurora A-CENP-F axis and the R loop-RPA axis for ATR activation may ensure that ATR is specifically activated in prometaphase at centromeres. The local activation of ATR at centromeres does not appear to affect CDK1 in mitosis, possibly due to the restriction of p-Chk1 to centromeres and the stabilization of CDC25A by CDK1 (25, 26), Instead, ATR activates Chk1 locally at centromeres to promote Aurora B activation, ensuring accurate chromosome segregation in mitosis.

This study uncovers an R loop-driven signaling pathway that promotes accurate cell division. Although R loops cause genomic instability in S phase, they are necessary for faithful mitosis. The opposite effects of R loops on genomic stability during S phase and mitosis create a demand for cell cycle-regulated ATR responses. Through two distinct ATR pathways, ATR suppresses R loopassociated genomic instability in S phase but mediates the centromeric function of R loops in mitosis (fig. S18). The activation and function of ATR at DNA breaks and replication forks are regulated by DNA repair and replication proteins. The ability of the ATR circuitry to rewire in a context-dependent manner may allow it to play an integral role at centromeres in mitosis distinct from its other functions. Both the genomic instability in S phase (replication stress) and the chromosome instability in mitosis (CIN) are hallmarks of cancer and vulnerabilities of cancer cells (27-29). The dual functions of ATR in countering replication stress and CIN may make ATR a particularly attractive therapeutic target in cancers with both vulnerabilities.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/108/suppl/DC1 Materials and Methods Figs. S1 to S18 Reference (30)

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IMMUNOLOGY

S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense

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Innate lymphoid cells (ILCs) are innate counterparts of adaptive T lymphocytes, contributing to host defense, tissue repair, metabolic homeostasis, and inflammatory diseases. ILCs have been considered to be tissue-resident cells, but whether ILCs move between tissue sites during infection has been unclear. We show here that interleukin-25- or helminth-induced inflammatory ILC2s are circulating cells that arise from resting ILC2s residing in intestinal lamina propria. They migrate to diverse tissues based on sphingosine 1-phosphate (S1P)-mediated chemotaxis that promotes lymphatic entry, blood circulation, and accumulation in peripheral sites, including the lung, where they contribute to anti-helminth defense and tissue repair. This ILC2 expansion and migration is ■ behavioral parallel to the antigen-driven proliferation and migration of adaptive lymphocytes to effector sites and indicates that ILCs complement adaptive immunity by providing both local and distant tissue protection during infection.

nnate lymphoid cells (ILCs) (1, 2) lack antigenspecific receptors but, upon suitable stimulation, produce effector cytokines that parallel those made by antigen-induced Thelper subsets (3). Various ILC progenitors have been identified in fetal liver and bone marrow (4), whereas mature ILCs are abundant in mucosal tissues and provide immune protection against pathogens early in infection (5-8). ILCs are also found in nonmucosal sites, such as secondary lymphoid tissues, and can mediate the transition from innate to adaptive immune responses (9, 10) while also playing important roles in epithelial

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tissue repair (11), fat metabolism (12, 13), and tumor immune surveillance (14). One issue that remains incompletely explored involves the specific origin(s) of ILCs present in diverse tissue sites and whether mature ILCs move between sites when infection demands. This is of special importance in the case of ILC2s and helminthic infections, because the latter typically involve different tissues such as the intestinal tract and lungs. Here we show that inflammatory ILC2s (iILC2s) (15) induced by helminths or the cytokine interleukin-25 (IL-25) migrate between tissues in response to activating signals. We also show how this migration is mediated and demonstrate the important role of such interorgan trafficking in host defense.

To probe the origin of tissue-resident ILCs, we monitored their numbers in the lung and small intestine of mice from postnatal day 1 through adulthood. On day I, a few hundred GATA-3hi ILC2s and RORyt⁺ ILC3s each were detected in the lungs (fig. S1A) and small intestine (fig. S1B). ILC2s, but not ILC3s, dramatically increased in number in the lung in the first week after birth and became the dominant ILC population, expanding further over the subsequent 3 weeks (fig. S1A). The greatest increase in ILCs in the

ILCs are generally considered to be tissueresident cells (16, 17). To further study ILC localization in the steady state and during infection. parabiotic mice were used. Given the substantial effects of commensal bacteria on the host immune system, antibiotics were administered for only 2 weeks after surgery. A 50:50 exchange rate in blood leukocytes was observed 1 week after surgery (fig. S2A), and lung and intestinal CD4 T cells showed exchange rates of 50:50 and 30:70 to 40:60, respectively, 2 months after surgery (fig. S2B). In contrast, lung ILC2s and intestinal ILC3s did not appreciably exchange between the two partners even 6 to 8 months after surgery (Fig. 1, A and B), suggesting that these ILC subsets are largely self-maintained and that progenitors in bone marrow contribute little to their numbers in the steady state. Although intestinal ILC2 exchange was barely detected at 2 months, a modest but significant increase to a ~10% exchange rate was observed at 6 to 8 months after surgery (Fig. 1B). ILC2s in mesenteric lymph nodes (MLNs) showed a 20% exchange rate, whereas ILC3s in the MLNs did not exchange (Fig. 1C). Thus, although other ILC2 or ILC3 subsets appear to be self-maintained, intestinal ILC2s are refreshed at a low rate in the steady state.

We recently reported the existence of a lineagenegative (Lin-), KLRG1hi ILC2 population that is induced in the lung, liver, MLNs, and spleen after treatment with IL-25 or inoculation with infective third-stage Nippostrongylus brasiliensis larvae (L3) (15). These iILC2s are distinct from the ILC2s that naturally reside in the lung (nILC2s) (18). However, the source of iILC2s in various organs remained undetermined. Thus, we investigated ILC2 activation and migration in response to IL-25 treatment. Intranasal administration of IL-25 did not elicit iILC2s in the lung, whereas intraperitoneal (i.p.) injection of IL-25 induced the appearance of KLRG1hi ST2-iILC2s (Fig. 1D). suggesting the absence of iILC2 precursors in the lungs. In vivo antibody labeling was performed in CD45.1⁺ CD45.2⁺ mice treated with IL-25. nILC2s were not labeled acutely by injected antibodies, indicating that they reside in parenchymal lung tissue. In contrast, like CD4 T cells, a large number of iILC2s were labeled, suggesting that they exist in the vascular space and circulate in the blood stream (Fig. 1E).

A parabiotic mouse model was used to further address the issue of iILC2 recirculation. After IL-25 i.p. injection into the CD45.1 mouse of a parabiotic pair, KLRG1hi iILC2s were found in the lungs of both the CD45.1+ mouse and its CD45.2⁺ partner (Fig. 1F). Notably, the majority of iILC2s in the lung and liver, 50% of iILC2s in the spleen, and 25% of iILC2s in MLNs in the CD45.2⁺ mouse were derived from its CD45.1⁺ partner (Fig. 1F and fig. S3, A to C), indicating that they are circulating cells. In contrast, ThyI^{hi} nILC2s in the lung were endogenously derived (Fig. 1F), suggesting that they did not circulate upon IL-25 treatment. Very few CD45.2+ iILC2s

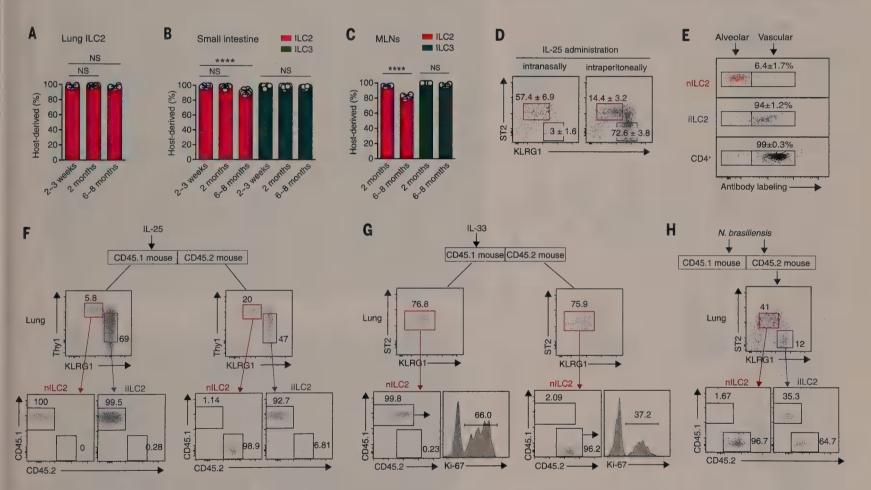


Fig. 1. IL-25- or helminthic infection-induced inflammatory ILC2s are circulating cells that differ from tissue-resident ILCs. (A to C) CD45.1+ and CD45.2⁺ mice were surgically connected to generate parabiotic partners. Percentages of host-derived cells were analyzed among ILC2s and ILC3s 2 to 3 weeks, 2 months, and 6 to 8 months after surgery. ILC2s were gated as lineage-negative (Lin⁻) CD127⁺ GATA-3^{hi}, and ILC3s were gated as Lin CD127 RORyt. MLNs, mesenteric lymph nodes. (D) B6 mice were treated with IL-25 intranasally or intraperitoneally (i.p.) daily for 3 days. Lung leukocytes were then analyzed by flow cytometry. Cells were gated on CD45.2+ Lin-CD127+. (E) CD45.1+ CD45.2+ mice were treated with IL-25 i.p. daily for 3 days. Mice were intravenously injected with phycoerythrin-labeled antibodies against CD45.1 and euthanized

5 minutes later for analysis of CD45.2 on Lin⁻ CD127⁺ ST2⁺ KLRG1^{int} naturally lung-residing ILC2s (nILC2s), Lin⁻ CD127⁺ ST2⁻ KLRG1^{hi} inflammatory ILC2s (ilLC2s), and CD4⁺ Tcells in the lungs without perfusion. (F and G) Two months after surgery, the CD45.1+ partner of each parabiotic pair was treated i.p. with IL-25 (F) or IL-33 (G) daily for 3 days. Cells in the lungs were then analyzed. nlLC2s were gated as Lin⁻ CD127⁺ Thy1^{hi} KLRG1^{int}, and ilLC2s were gated as Lin⁻ CD127⁺ Thy1^{lo} KLRG1^{hi}. (**H**) Two months after surgery, both mice in each parabiotic pair were inoculated with ~200 infective N. brasiliensis larvae (L3). Cells in the lungs were analyzed on day 5 postinoculation. Means ± SEM from six to eight mice at each time point in (A) to (C) and from three mice in (D). ****P ≤ 0.0001; NS, not significant; unpaired two-tailed t test. Results are representative of at least two independent experiments.

were found in the lung and liver (Fig. 1F and fig. S3A), possibly owing to the short half-life of exogenous IL-25 circulating via the blood to the partner mouse. Although IL-33 treatment induced lung nILC2 proliferation in both animals (Fig. 1G), based on increased Ki-67 expression (fig. S1C), nILC2 transfer between the parabiotic animals did not occur, indicating that these cells do not enter the circulation. Consistent with the IL-25 treatment data, 5 days after the inoculation of both parabiotic partners with N. brasiliensis L3, 35% of iILC2s in the lung were derived from the partner mouse, whereas the majority of nILC2s were endogenous (Fig. 1H). Thus, IL-25- or N. brasiliensis-induced iILC2s are circulating cells, distinct from tissue-resident ILC subsets.

To identify the source of circulating iILC2s, total leukocytes were isolated from different CD45.1⁺ $RagI^{-/-}$ mouse tissues and then transferred into CD45.2⁺ $RagI^{-/-}$ mice; this was followed by IL-25 treatment. Unexpectedly, cells

from small intestine lamina propria (siLP) gave rise to high numbers of rapidly proliferating iILC2s in the lungs of the recipients, whereas transferred bone marrow cells gave rise to few iILC2s, and lung cells gave rise to none (Fig. 2A). This suggested that the intestine contains an enriched source of "pre-iILC2s." siLP leukocytes were then divided into three groups, and similar cell transfer experiments were performed. Only KLRG1⁺ ILC2s from the siLP could give rise to iILC2s in the lung of recipients—neither Lin⁺ cells nor KLRG1- ILCs could do so (Fig. 2B)-suggesting that IL-25-induced iILC2s in peripheral sites are derived from intestinal ILC2s. In addition, we purified lung nILC2s, bone marrow (BM) ILC2 progenitors, and siLP ILC2s and transferred equal numbers of these populations to recipients; we then administered IL-25 treatment. Intestinal ILC2s were much more efficient in giving rise to iILC2s than were BM ILC2 progenitors. Most iILC2s in the recipients were Ki-67⁺, indicating

that they were proliferating rather than just phenotypically converted from donor cells (Fig. 2C). Although BM ILC2 progenitors may contribute to peripheral responses in chronic conditions, they are unlikely to be the major source of iILC2s during acute helminthic infections, owing to the lack of local IL-25 producers such as the tuft cells of the gastrointestinal tract (19–21).

Flow cytometry revealed that IL-25-induced iILC2s and siLP ILC2s possess similar surface-marker phenotypes (Fig. 2D). To further characterize ILC2 populations, we examined the transcriptomes of BM ILC2 progenitors, lung nILC2s, IL-33-activated lung nILC2s, intestinal IILC2s, IL-25-induced lung iILC2s, and MLN iILC2s by RNA sequencing (RNA-seq). Despite the difference in location, lung and MLN iILC2s have very similar transcriptome profiles (Fig. 2E). Intestinal ILC2s showed the closest resemblance in gene expression pattern to iILC2s, providing additional evidence that the source of IL-25-induced

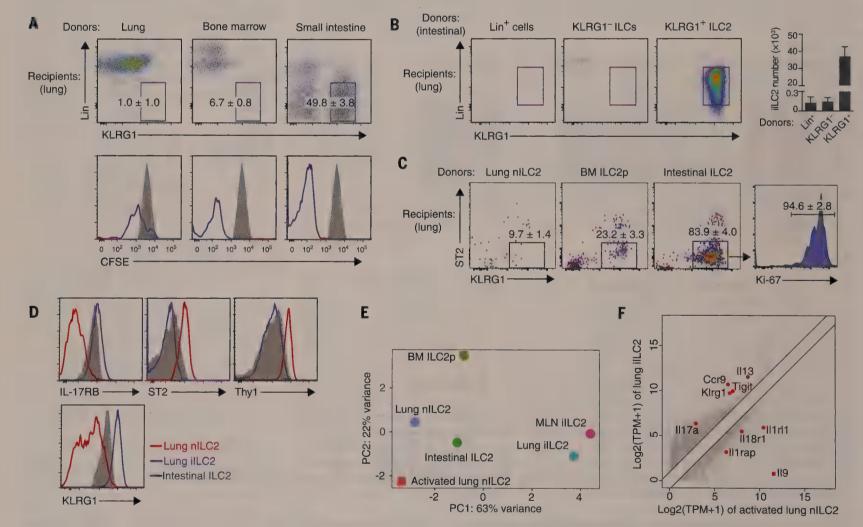


Fig. 2. Intestinal ILC2s are the immediate source of iILC2s. (**A**) Total leukocytes from lung, bone marrow, or siLP of CD45.1⁺ $Rag1^{-/-}$ mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred into CD45.2⁺ $Rag1^{-/-}$ mice; this was followed by a 3-day treatment with IL-25 injected i.p. Cells in the lungs were analyzed for CD45.1⁺ donor cells. Blue line, Lin⁻ KLRG1^{hi} iILC2s; gray shaded area, CD45.1⁺ cells labeled with CFSE before transfer. (**B**) siLP leukocytes were divided into the three indicated groups by cell sorting and subjected to a similar transfer experiment to that described in (A). Left, cells were gated on CD45.1⁺ Lin⁻; right, CD45.1⁺ iILC2 numbers in the lungs. (**C**) Equal numbers of lung nILC2, siLP ILC2, and bone marrow (BM) ILC2 progenitors were sorted and subjected

to a similar transfer experiment to that described in (A). (**D**) Comparison of surface markers on lung ilLC2s in IL-25—treated mice and lung nlLC2s and intestinal ILC2s in naïve mice by flow cytometry. (**E**) Principal component analysis of the transcriptomes of different ILC2 populations identified by RNA-seq. The analysis is based on $\log_{10}[\text{transcripts per million (TPM)} + 1]$ of 53 immune-related genes. (**F**) Scatter plot comparing the gene expression patterns from RNA-seq between activated lung nlLC2s and lung ilLC2s. The x and y axes show the $\log_{10}(\text{TPM} + 1)$ values for each sample. Only genes with at least a twofold difference were plotted. Means \pm SEM from three mice in (A) to (C). Data are representative of two independent experiments in (A) to (D).

iILC2s is intestinal ILC2s. Activated lung nILC2s and lung iILC2s showed highly distinct gene expression patterns, although they were in the same tissue (Fig. 2F). iILC2s produce more IL-13, whereas nILC2s produce more IL-9. We also found that iILC2s express high levels of CCR9 and some inhibitory receptors such as KLRG1 and TIGIT. In addition, we found that iILC2s produce IL-17A, which is consistent with our previous findings (15), whereas nILC2s express a higher level of the receptor (IL-18R1) and receptor-associated protein (IL-1RAcP) involved in responses to IL-18 and IL-1 (Fig. 2F), cytokines that are important for the conversion of lung ILC2s into ILC1s (22-24).

iILC2s appear in the lungs during the early stage of pulmonary N. brasiliensis larval migration and disappear after the expulsion of adult worms from the intestine (15). In parabiotic mouse experiments, KLRG1hi iILC2s were no longer detected in the lungs 12 days after a 3-day IL-25 treatment. However, among lung nILC2s in the

untreated CD45.2⁺ mouse partner, ~10% were CD45.1⁺ (fig. S4A). Among lung nILC2s, ~16% were derived from the parabiotic donor 20 days after inoculation of N. brasiliensis into both partners (fig. S4B), suggesting that iILC2s can contribute to the lung nILC2 pool late in infection. Notably, among siLP ILC2s of the CD45.2+ mouse, ~40% were CD45.1+ (fig. S4A). iILC2s were found to express high levels of integrin $\alpha 4\beta 7$ (25) and gut-homing receptor CCR9 (26) (fig. S4C), suggesting that iILC2s also have the potential to return to the gut after infection. CD62L expression was detected on iILC2s in the MLNs, but not in the lung. Thus, iILC2s present in peripheral sites are a transient cell population, converting into nILC2s in tissues such as the lung and/or migrating back to the gut.

To gain mechanistic insight into the effects of IL-25 on siLP pre-iILC2s and the control of their exit into the circulation, we used confocal imaging. The coexpression of GATA-3 and KLRG1

confirmed that KLRGI is a reliable marker for intestinal ILC2s (fig. S5). In the steady state, CD3 KLRG1 ILC2s predominantly resided in the lamina propria, and only a small fraction were Ki-67⁺ (Fig. 3A, left). Thirty-six hours after IL-25 treatment, more than 50% of ILC2s were Ki-67⁺. Sixty hours after IL-25 treatment, ILC2 numbers dramatically increased, and the majority were Ki-67⁺ (Fig. 3, A and B). Intestinal ILC2s expressed substantial levels of cell-surface CD69, whereas levels in circulating iILC2s were lower (Fig. 3C). CD69 has an important role in the control of T cell migration between tissues. It is highly expressed on tissue-resident memory T cells, prolonging their residency, and on activated T cells in lymph nodes during the sequestration phase shortly after the initiation of an immune response. In contrast, its expression on trafficking T cells is low (27). Given that KLRG1hi ILC2s were present in many peripheral sites 60 hours after IL-25 administration (fig. S3C), we hypothesized that

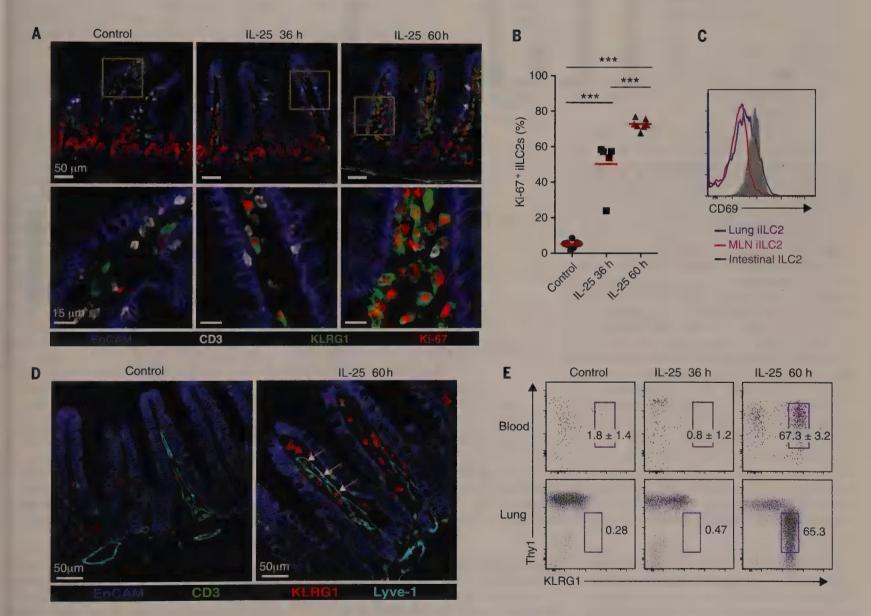
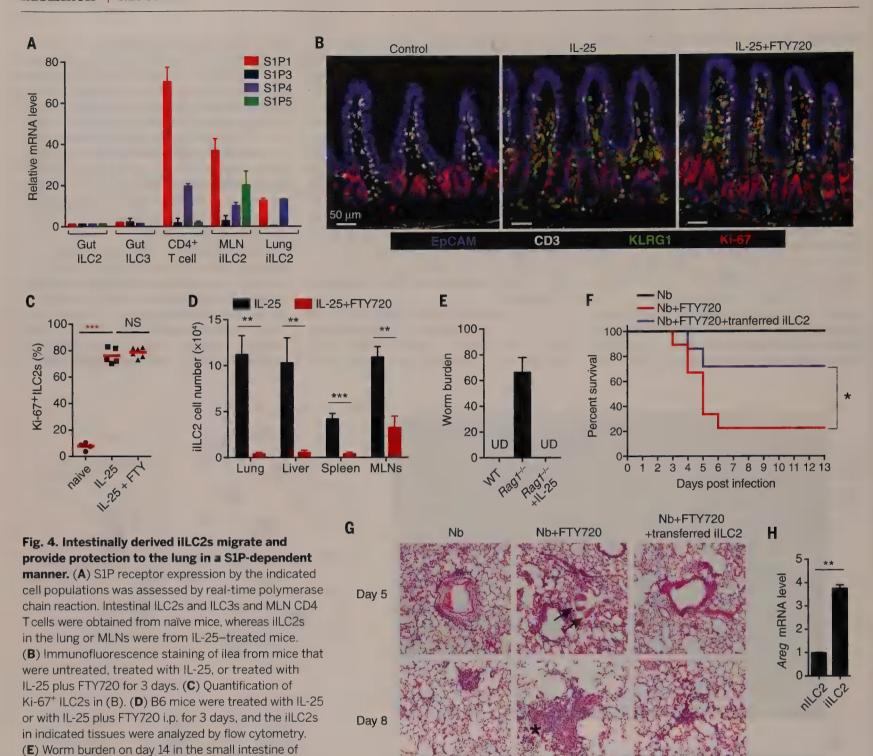


Fig. 3. Intestinal ILC2s proliferate and enter lymphatics in response to IL-25. (A) Immunofluorescence staining of ilea from B6 mice 0, 36, and 60 hours (h) after treatment with IL-25. The lower panels are magnified views of the boxed areas in the upper panels. (B) Quantification of Ki-67⁺ ILC2s in the small intestine. (C) Comparison of CD69 expression on siLP ILC2s, lung iILC2s, and MLN iILC2s by flow cytometry. (D) Immunofluorescence staining of ilea from mice 0 and 60 hours after

treatment with IL-25. Arrows, KLRG1+ ILC2s within a lymphatic vessel. (E) ilLC2s in peripheral blood or lungs were analyzed from B6 mice 0, 36, and 60 hours after treatment with IL-25. Images are representative of three gut sections from two mice in each group. Data are representative of two independent experiments in (C) and (E). Means \pm SEM; n = 6 to 9 and 3 for each group in (B) and (E), respectively. *** $P \le 0.001$; unpaired two-tailed t test.



treatment was given every other day in the third group.

UD, undetected. (**F**) Rag1^{-/-} mice were inoculated with ~500 N. brasiliensis L3 (Nb), and FTY720 treatment started on day 3 and continued daily until day 10, in two groups. In the transfer group, ~3 × 10⁶ ilLC2s sorted from IL-25—treated mice were transferred intravenously into each animal on the same day as worm inoculation. Death and survival were monitored until day 13, and the difference between two groups was determined by the log-rank (Mantel-Cox) test. (**G**) Histology of lung sections from Rag1^{-/-} mice inoculated

with ~300 *N. brasiliensis* L3. Arrows, worm larvae; asterisk, tissue destruction. **(H)** Amphiregulin mRNA levels in nlLC2s and ilLC2s, which were sorted from lungs on day 5 postinoculation with *N. brasiliensis*. Means \pm SEM; n=3 in (A), (D), (E), and (H) and 4 to 6 in (C). * $P \le 0.05$; ** $P \le 0.01$; *** $P \le 0.001$; unpaired two-tailed t test. Data in (A), (D), and (E) are representative of three experiments; data in (F) are the summary of two experiments (n=10). Images are representative of three sections from two mice of each group in (B) and (G).

activated intestinal ILC2s behave like activated T cells in lymph nodes, crossing the lymphatic endothelium, entering lymphatics, and entering the blood circulation. When Lyve-1 staining was used to delineate lymphatic vessels (28), KLRG1⁺ ILC2s were observed within these vessels in the villi of IL-25-treated mice but not of naïve mice (Fig. 3D). Three-dimensional image reconstruction confirmed the localization of ILC2s within, rather than adjacent to, the lymphatic vessels (movie S1). iILC2s were also detected in peripher-

al blood 60 hours after IL-25 treatment (Fig. 3E). Thus, intestinal ILC2s rapidly proliferate after IL-25 stimulation and enter lymphatic vessels and then the blood, accumulating as iILC2s in many peripheral sites.

G protein-coupled sphingosine 1-phosphate (S1P) receptors are required for T lymphocyte egress from lymphoid organs across lymphatic endothelial barriers (29, 30). Thus, we examined the possible role of this chemotactic pathway in ILC migration. ILC2s or ILC3s in naïve mice did

not express S1P receptors, but IL-25-induced iILC2s in the lung and MLNs, like CD4 T cells, expressed S1PR1 and S1PR4 (Fig. 4A). iILC2s in MLNs also expressed S1PR5 (Fig. 4A), which has been reported to be expressed on natural killer cells (31). FTY720, which antagonizes the S1P-signaling pathway, did not affect IL-25-induced intestinal ILC2 proliferation (Fig. 4, B and C) but blocked iILC2 accumulation in the lung, liver, and spleen and partially blocked iILC2 accumulation in MLNs (Fig. 4D). This is consistent with

the tissue-specific differences in CD69 expression (Fig. 3C) (27, 32). Together, these results indicate that iILC2 cells use a similar molecular mechanism to that of conventional CD4+ and CD8+ T cells, which regulates their exit from tissues into the lymph as they move to a distant site of effector activity.

IL-25R+ ILC2 progenitors were recently identified in bone marrow (33). Thus, we addressed the relationship between BM ILC2 progenitors and iILC2s. IL-25R⁺ progenitors expressed ST2 but not KLRG1 in naïve mice (fig. S6, A and B). IL-25 treatment not only expanded the progenitor population, but also induced ST2- KLRG1hi iILC2s in the bone marrow (fig. S6B). FTY720 did not affect the expansion of ILC2 progenitors but abolished the presence of iILC2s in bone marrow (fig. S6, B and C). These results indicate that intestinally derived iILC2s can infiltrate the bone marrow and highlight the distinction between iILC2 and BM ILC2 progenitors.

Last, we investigated the physiological implications of ILC2 migratory responses during antihelminth immunity. Inoculation of conventional $RagI^{-/-}$ mice with N. brasiliensis established a chronic adult worm infection in the intestine, which was cleared by the administration of IL-25 (Fig. 4E), suggesting that an increase in iILC2 numbers is sufficient to expel the worms even in the absence of adaptive lymphocytes. Parasitic N. brasiliensis L3 pass through the lungs early in the infection and cause inflammation and tissue damage. FTY720 treatment of Rag1^{-/-} mice inoculated with a dose of 500 L3 resulted in the death of 80% of the mice at early stages of infection (Fig. 4F). This mortality was largely prevented by the transfer of iILC2s into the circulating pool before FTY720 treatment, which compensated for the drug-induced blockade of endogenous iILC2s in the lung. Larvae were observed in the lungs (arrows) on day 5, and severe epithelial destruction was noted (asterisks) on day 8 postinoculation in FTY720-treated mice not given adoptive cell therapy (Fig. 4G). Prolonged worm residency and tissue damage could be prevented by iILC2 transfer. Furthermore, iILC2s were found to express higher levels of amphiregulin—a key contributor to epithelial tissue repair during infection—compared with nILC2s (Fig. 4H). Thus, intestinally derived iILC2s accumulate in the lung in a S1P-dependent manner and provide crucial protection at the early stage of infection, contributing to worm clearance, tissue repair, and host survival.

Here we have characterized in detail a distinct cell population that resides in the gut but can migrate to the lung and other distal sites and make substantial contributions to host defense. Tissue-resident ILC2s undergo interorgan migration, a property essential to their protective role in infection. This study also shows that epitheliumresident ILC2s move into lymphatics in a S1Pdependent manner by the same mechanism previously described for adaptive lymphocytes egressing from secondary lymphoid tissues. We hypothesize that SIP-dependent dissemination of activated effectors from one tissue through the lymphatics and blood to a distant site of infection evolved as a mechanism within the innate lymphoid system, which was later grafted onto the emerging adaptive system, rather than being a late development of the T cell adaptive immune system. Last, the ability of FTY720 to block ILC2 dissemination suggests that the immunosuppressive effects of this drug that have been attributed solely to the blockade of adaptive T cell migration from lymph nodes and the spleen need to be reassessed.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6371/114/suppl/DC1 Materials and Methods Figs. S1 to S6 References (34-36)

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The **Biotechnology Centre** (BIOTEC) (www.biotec.tu-dresden.de), an institute at the Center for Molecular and Cellular Bioengineering (CMCB), developing innovative technologies and approaches for basic and applied research and teaching in the modern life sciences, with particular strengths in molecular cell- and developmental biology, physical biology, and computational biology, offers a

Chair (W3) of Cellular Biochemistry

starting at 1. October 2018.

The successful applicant will represent the field of Cellular Biochemistry in research and teaching. Her/His research should integrate well with and complement the interdisciplinary research at the BIOTEC and the wider Dresden research campus (e.g., the Center for Regenerative Therapies Dresden, the Center for Molecular Bioengineering (B CUBE), the School Science-and the Carl Gustav Carus Faculty of Medicine, as well as the Max Planck Institute of Molecular Cell Biology and Genetics. Teaching (in English) is possible and expected in the CMCB's international Master programmes in "Molecular Bioengineering", "Nanobiophysics", and "Regenerative Biology and Medicine", in the Bachelor programme "Molecular Biotechnology" of the Faculty of Biology, and in the Dresden International PhD program DIPP. The duties include participation in academic self-administration

We are seeking outstanding applicants with an international scientific reputation in the field of Cellular Biochemistry. Research could be in any area of Cellular Biochemistry. A focus could be for example on the biochemical basis of forming macromolecular assemblies inside the cell, the role of weak biomolecular interactions in intracellular organisation, or dynamic regulatory processes leading to pattern formation in early development, including the setup of neuronal circuitry. An excellent publication track record and a proven ability to attract significant third-party funding are required. Applicants must fulfil the employment qualification requirements of § 58 of the Act on the Autonomy of Institutions of Higher Education in the Free State of Saxony (Higher Education Act of the Free State of Saxony — SächsHSFG). She/He must have a doctoral degree in natural sciences and a habilitation or an equivalent record of outstanding research achievements.

For further information please call +49 351 463-40054

TU Dresden seeks to employ more female professors. Hence we particularly encourage women to apply. Applications from disabled candidates or those with additional support needs are very welcome. The University is a certified family-friendly university and offers a dual-career service. If you have any questions about these topics, please contact the Equal Opportunities Officer of CMCB (Martin Kaßner, +49 351 458-82082) or the Representative of Employees with Disabilities (Ms Birgit Kliemann, +49 351 463-33175).

Applications containing CV, publication list, third-party funding acquired and a no more than five pages long description of past research achievements, teaching experience as well as the future research and teaching concept should be sent until 05.02.2018 (stamped arrival date applies) to: TU Dresden, Biotechnologisches Zentrum, Direktor, Herrn Prof. Jochen Guck, Tatzberg 47/49, 01307 Dresden, Germany and as electronic copy via TU Dresden's SecureMail Portal https://securemail.tu-dresden.de by sending it to dana.schoder@tu-dresden.de. In addition, please arrange to have two confidential letters of recommendation sent on your behalf to the address above.





新淘大學腦研究所

BRAIN RESEARCH INSTITUTE, NIIGATA UNIVERSIT

Job Opening

Professor in Cellular Neuropathology Brain Research Institute, Niigata University, Japan

Brain Research Institute, Niigata University is currently seeking a full professor who explores system neuropathology and neurobiology for brain diseases, applying molecular biology, cellular biology, mouse engineering and/or neurophysiology. Candidates who have a potential to lead the given neuroscience field are desirable.

1. JOB DETAILS

- Position
 A full professor (Tenured. Salary will be commensurate with experience)
- 2. Employment starting date
 Anticipated starting date is April 2018, or as soon as possible
 thereafter

2. APPLICATION PROCEDURE

Interested candidates are encouraged to apply by email and should submit as PDF documents:

- 1. A Curriculum Vitae
- 2. List of achievements
- 3. Reprints of main research publications (5 papers)
- 4. Research interests and accomplishments, teaching experience, and a research plan
- 5. References (at least 2)

3. CLOSING DATE

January 31, 2018 at 5:00 pm (JST)

To apply or make inquiries, please email Brain Research Institute, Niigata University, at noukenshomu@adm.niigata-u.ac.jp

Please refer to the following webpages for more information: http://www.bri.niigata-u.ac.jp/en/info/officialannounce/000960.html



SEEKING NOMINATIONS FOR TRANSLATIONAL RESEARCH PRIZE

Indiana University School of Medicine is accepting nominations for the 2018 August M. Watanabe Prize in Translational Research, awarded to an investigator who has made a significant contribution to the field of translational science.

The Watanabe Prize is one of the nation's largest and most prestigious awards recognizing individuals focused on shepherding scientific discoveries into new therapies for patients. It is named in honor of the late August Watanabe, a titan in the field of translational research in both academia and industry, who impacted the health of people around the world as ■ leader at IU and Eli Lilly and Company.

Nominees for the Watanabe Prize should be members of the scientific or medical community who have achieved outstanding accomplishments in translational research. This award is conferred upon senior investigators whose influential research deserves major recognition.

The winner of the 2018 Watanabe Prize will receive a \$100,000 award and spend time in Indianapolis as a visiting dignitary to share knowledge with audiences at IU and partner institutions. Over the next two years, the honoree also will serve as a long-distance mentor to two exceptional young investigators named concurrently as Watanabe Translational Scholars. Note: This year's prize nominees must be available to travel to Indianapolis from September 12-14, 2018.

To submit a nomination, email amwprize@iu.edu with: 1) a letter of nomination including a detailed description of the nominee's major translational research accomplishments and their impact, and 2) a copy of the nominee's current curriculum vitae (CV) by January 31, 2018. Questions? Contact amwprize@iu.edu or 317-278-2874.

Indiana University is an equal opportunity employer committed to building a culturally diverse intellectual community and strongly encourages applications from women and minorities.

UW Medicine

UW SCHOOL OF MEDICINE

Professor and Chair, Department of Microbiology, University of Washington

The University of Washington, School of Medicine seeks a Professor and Chair of the Department of Microbiology. Candidates for this full time position should have a PhD and/or an MD (or foreign equivalent) in Microbiology or a related biomedical discipline, an internationally recognized research program in Microbiology and demonstrated academic leadership skills. The Department of Microbiology has outstanding research and training programs, including an undergraduate major and participation in both departmental and interdisciplinary graduate programs (https://microbiology.washington.edu). The Department is ranked #3 worldwide in microbiology by *U.S. News and World Reports* and is an integral part of a broad, vigorous research community. The UW School of Medicine is #2 in the nation and #1 among public universities in NIH funding. UW faculty engage in teaching, research and service while training the next generation of scientists through undergraduate, graduate and medical student education.

Please submit an application (including a cover letter addressed to Dr. Joan Goverman, Chair, Microbiology Chair Search Committee and your curriculum vitae) at http://apply.interfolio.com/47603.

Applications will be considered until the position is filled.

University of Washington is an Affirmative Action and Equal Opportunity Employer. All qualified applicants will receive consideration for employment without regard to race, color, religion, sex, sexual orientation, gender identity, gender expression, national origin, age, protected veteran or disabled status, or genetic information.

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Head and Professor Department of Biological Sciences Purdue University



Purdue University invites applications for the position of Head of the Department of Biological Sciences to start August 2018. The department seeks a dynamic leader with creative vision and an outstanding record of research, administration, and teaching.

The department is undergoing a period of exciting development. Departmental strengths include Biology Education; Cell & Molecular Biology; Ecology and Evolutionary Biology; Microbiology & Infectious Disease; Neuroscience & Physiology; and Structural Biology. Many departmental faculty are involved in university-wide multidisciplinary research through Discovery Park (www.discoverypark.purdue.edu) and the Purdue Center for Cancer Research (www.cancerresearch.purdue.edu). In addition, members of the department play leading roles in the recently launched Purdue Institute for Integrative Neuroscience http://www.purdue.edu/discoverypark/pillars/integrative-neuroscience-center/ and Purdue Institute of Inflammation, Immunology and Infectious Disease https://www.purdue.edu/discoverypark/pillars/pi4d/. As of Fall 2017, the Biological Sciences Department is comprised of 55 faculty members, 27 post-doctoral researchers, 118 graduate students, and 899 undergraduate biology majors. Further information about the department is available at www.bio.purdue.edu.

Qualifications: The successful candidate will have: a Ph.D. in Biology or a related discipline; an outstanding record of scholarly achievement and a history of extramurally funded research commensurate with the rank of full professor at Purdue; dedication to building a diverse, inclusive, and vibrant scientific community, exceptional and proven leadership abilities; an effective vision for the department in the university, state, and nation; commitment to excellence in undergraduate and graduate education; and an enthusiasm for engagement.

Applications: We encourage qualified candidates to apply online at http://hiring.science.purdue.edu. Inquiries should be directed to Biology Head Search Committee, Department of Biological Sciences, Purdue University, 915 W. State St., West Lafayette, IN 47907-2054 or search@bio. purdue.edu. Purdue University's Department of Biological Sciences is committed to advancing diversity in all areas of faculty effort, including scholarship, instruction and engagement. Candidates should address at least one of these in their cover letter, indicating their past experiences, current interests or activities, and/or future goals to promote a climate that values diversity and inclusion. Review of applications will begin January 8, 2018 and will continue until the position is filled.

A background check is required for employment in this position. Purdue University is an EEO/AA Employer. All individuals, including minorities, women, individuals with disabilities, and veterans are encouraged to apply.



THE UNIVERSITY OF TEXAS AT DALLAS

School of Natural Sciences and Mathematics

DEPARTMENT OF BIOLOGICAL SCIENCES

ASSISTANT PROFESSOR

The Department of Biological Sciences in the School of Natural Sciences and Mathematics at the University of Texas at Dallas (http://www.utdallas.edu/biology/) invites applications for a faculty position at the rank of Assistant Professor. We seek to hire an outstanding scientist with research interests that complement and build on existing departmental strengths in biochemistry, cell and molecular biology, computational and systems biology, genomics, microbiology, and pathobiology (cancer, neurodegenerative disorders, and infectious disease). Since the department has the goal of expanding its research and teaching into new directions, we will also consider applications from excellent candidates in other areas of the biological sciences. The University has recently made a major investment in new laboratory space, and in core facilities (genomics, imaging and histology, flow cytometry, and protein analysis) that will be available to support the research of new recruits.

Applicants should be prepared to establish a vigorous and independent research program and should have enthusiasm for teaching at both graduate and undergraduate levels.

Review of applications will begin immediately and will continue until the position is filled. Indication of sex and ethnicity for affirmative action statistical purposes is requested as part of the application but is not required.

Application materials: cover letter, curriculum vitae, short descriptions of research plans and teaching interests and the full contact information for at least three references should be submitted at: http://jobs.utdallas.edu/postings/9165.

The University of Texas at Dallas is an Equal Opportunity/ Affirmative Action employer and strongly encourages applications from candidates who would enhance the diversity of the University's faculty and administration.



UNIVERSITY

Systems, Molecular, and Cellular Mechanisms of Alzheimer's **Disease and Dementia**

THE OHIO STATE The Ohio State University is launching a major initiative focused on the systems, molecular, and cellular mechanisms of

Alzheimer's disease and adult-onset dementia. To this end, the University is recruiting 15 faculty to multiple departments at all ranks to study injury-induced dementia and Alzheimer's disease. As part the initiative, The Department of Neuroscience in the College of Medicine is currently recruiting four or more tenure-track faculty at all ranks, in association with the OSU Discovery Theme in Chronic Brain Injury. Candidates must hold a PhD or MD (or equivalent) with research expertise in areas directly related to mechanisms of Alzheimer's Disease or related disorders. This recruitment is part of an ongoing commitment to develop distinguished research and clinical care centers at The Ohio State University focused on adult-onset dementias.

Successful candidates are expected to contribute to the missions of the University, Department, and Centers via active participation in research and teaching programs, mentoring of trainees and serving on departmental or college-level committees. Salary will be competitive and commensurate with experience.

Prospective candidates should send a statement of research interests, vitae and a list of three references to Melissa Stenger (Melissa.stenger@osumc.edu). Consideration of candidates will begin on 1 January 2018.

Unless confidentiality is requested in writing, information regarding the applicants must be released upon request. For more information about career opportunities, please visit www.Discovery.osu.edu or https://wexnermedical. osu.edu/neurological-institute/departments-and-centers/departments/ department-of-neuroscience

The Ohio State University is an Equal Opportunity, Affirmative Action Employer and as such, women and underrepresented individuals in science are encouraged to apply.

WORKING LIFE

By Lydia Zepeda

The harassment tax

senior faculty member asked me into his office. I assumed it was to talk about agricultural data. It was the fall of 1991 and I was untenured, 32 years old, and 7 months pregnant. He was in his 60s and one of many men who were going to vote on my tenure. He showed me the recent issue of *Vanity Fair* with Demi Moore on the cover, pregnant and nude. "She reminds me of you," he said as he tried to catch my eye. I looked at the floor, stunned. I mumbled something and backed out of his office, wondering whether I would ever feel clean again.

This was just one example of the sexual harassment I experienced during my career as a professor. It happened to me; it happens to other female faculty members; and it happens to female staff, graduate students, and undergraduates. It wasn't all men and it didn't happen all the time, but it happened, and it was part of my life in academia: grant writing, teaching, publishing in peer-reviewed journals—oh, and dealing with creeps and the messes they made.

Sexual harassment is draining. It takes up time and energy, and it does not result in anything for one's CV or annual review. It is a productivity tax on women. In my case, it meant I avoided co-authoring or having joint grants with male colleagues, things that

would likely have increased my funding and publications.

The costs also spill over to others. By taking up women's energy and lowering productivity, harassment wastes valuable grant money and taxpayer funding. It is also a key reason women leave academia, which ultimately hurts the entire scientific enterprise in the form of lost investment, potential, and diversity of ideas. In my case, even though I was productive and loved research, teaching, and advising students, ubiquitous harassment was one of the reasons why I retired early.

Here are just a few examples of the sexual harassment I experienced that affected my productivity. A married colleague bragged to me about his sexual conquests. A junior colleague told me he wanted to date me. (I am married!) While I was interviewing for a full professor job, a department head in his 40s inquired how many children I had and, staring at my body, insisted that I "should get pregnant many more times." After I got tenure, the burden of harassment only increased. That's because I experienced it not just directly, but also secondhand, as other victims—students, staff members, colleagues, mentees—sought my help and time.

Recently, a graduate student confided in me that a



"Sexual harassment is ... a productivity tax on women."

renowned researcher had hit on her and touched her inappropriately during a postdoc interview. I advised her not to take the job because if he did that during the interview, in all likelihood it would escalate later. She would not be dissuaded. "It is just too good a job," she said. She decided to take a calculated risk because, she said, "what else are you going to do? It's everywhere." So, along with doing first-class research, she has to figure out how to keep her boss's hands off her.

Sexual harassment even affected my free time, interfering with my efforts to recharge and sustain my productivity. Earlier in my career, I played basketball with faculty and staff members on campus. I was usually the only woman. One

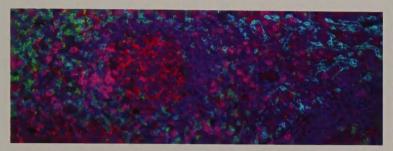
day, a man guarding me couldn't get the ball from me and punched me in the breast. It was hard enough to knock me to the floor and leave a bruise. When I demanded to know why he punched me, he yelled, "Women have no business here!" I wondered, did he mean playing basketball or being at the university?

I stopped playing basketball; dealing with harassment at work took enough energy and time. While I was angry at the man who assaulted me, I was angrier at the eight other men on the court. They all saw and heard what happened, yet they said and did nothing. They literally looked away. They may not have meant it, but to me their silence spoke volumes: approval.

It is time to speak up. We can start by having meaningful and transparent Title IX investigations that support, not attack or shame, victims. Speak up every time harassment happens. Men, call out other men. Every time. Show that you do not condone sexual harassment. Enough is enough. ■

Lydia Zepeda is a professor emeritus at the University of Wisconsin in Madison and a AAAS fellow. Send your career story to SciCareerEditor@aaas.org.

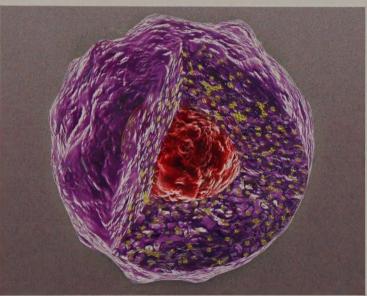
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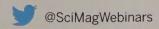


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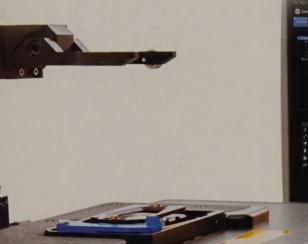
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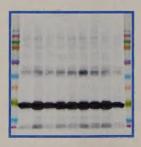
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